Process Characterization and Optimization for the Production of Bioactive Natural Products with Gliding Bacteria

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Abstract

Natural products experience an increasing importance in pharmaceutical research where the development of new lead compounds, especially those with antibiotic activity, has become imperative. The innovation gap left behind by the termination of natural product research in most pharmaceutical companies in the 1990s cannot be filled by chemically synthesized molecules, contrary to the belief of the past decades. In order to reinvigorate natural product development, the potential of so far neglected secondary metabolite producers like gliding bacteria from the order of Myxococcales and the genus *Chitinophaga* needs to be investigated more thoroughly. This thesis comprises three projects dealing with the development and optimization of fermentation processes for the production of secondary metabolites with antibiotic activity from these gliding bacteria with the aim of increasing their accessibility for further research.

The first project covers the production of the anti-MRSA compound family of elansolids by its native producer *Chitinophaga sancti* Fx7914. The focus was set on the production of elansolid A2, the starting compound for semi-synthetical medicinal chemistry approaches. The other important derivative was elansolid C1, which is formed through nucleophilic addition of anthranilic acid directly after biosynthesis. The production of elansolid C1 can thus serve as a model process for precursor-directed biosynthesis with the ultimate goal of facilitating structure-activity-relationship studies.

The other two projects deal with the process optimization of the heterologous production of two types of myxobacterial compound classes with the model strain *Myxococcus xanthus* DK1622. The class of polyketides is represented by the α-pyrone myxopyronin A whereas the non-ribosomal peptide synthetase-derived compounds are represented by the peptolide family of vioprolides. Process optimization was accomplished by improvement of the media composition and process parameters using statistical experiment design, as well as precursor-feeding. These studies lead to the aim of investigating *M. xanthus* as an expression platform for natural products in general.

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Symbols and Abbreviations

Symbols and Abbreviations	**Name and Unit**
A	Acylation Domain
ACP	Acyl Carrier Protein
AT	Acyltransferase
C	Condensation Domain
CER	Carbon Dioxide Emission Rate (mM h^{-1})
CTR	Carbon Dioxide Transfer Rate (mM h^{-1})
Cy	Cylcization
DH	Dehydratase
DNA	Desoxyribonucleic Acid
DO	Dissolved Oxygen
DoE	Design of Experiments
E	Epimerization
EM	Extension Module
ER	Enoyl Reductase
F	Formylation
FDA	Food and Drug Administration
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High Performance Liquid Chromatography
HTS	High Throughput Screening
k	Number of Steps of Experimental Reduction
KR	Ketoreductase
KS	Ketosynthase
μ	Growth Rate (h^{-1})
4-Maz	4-methyl azetidine carboxylic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
MT	Methyl Transferase

Symbols and Abbreviations	Name and Unit
MS	Mass Spectrometer
N	Number of Experiments
n	Number of Factors
NP	Natural Product
NRP	Non-ribosomal Peptide
NRPS	Non-Ribosomal Peptide Synthetase
OTR	Oxygen Transfer Rate (mM h^{-1})
Ox	Oxidation
PCP	Peptidyl Carrier Protein
PK	Polyketide
PKS	Polyketide Synthase
PMBN	Polymyxin B Nonapeptide
PPan	4'-phosphopantetheine
PPTase	4'-phosphopantetheinyl Transferase
R	Reduction
RAMOS	Respiratory Activity Monitoring System
RNA	Ribonucleic Acid
RNAP	DNA-dependent RNA-polymerase
SAR	Structure Activity Relationship
vvm	Volume per Volume per Minute

1 Theoretical Background

1.1 Natural Product Research Throughout History

1.1.1 Historical Use of Natural Products

Natural products (NPs) possessing pharmaceutical activity have ever since been used by humans to treat illness. Archaeological evidence indicates that medicinal plants were regularly used by people in prehistoric times. Some of the earliest written records made by the ancient Egyptians (4,000 BC-395 AD) deal with this subject [1]. But the use was limited to substances of plant, animal and mineral origin. Plant-based treatments were also commonly administered in ancient Greece, 3,000 through 1,500 years ago. The use of medicinal plants and remedies of animal and mineral origin is documented for ancient cultures all over the world, like the Aztec and Maya Indians and Chinese, Tibetan and Indian cultures. All these cultures share the usage of selected molds, plant extracts or animal excretions to treat infections [2, 3, 4].

The first visualization of bacteria with the help of a microscope was managed by Antoni van Leeuwenhoek in 1674 that enabled their scientific exploration [5]. But for 200 years the idea that these microscopic organisms produced substances that were pharmaceutically relevant to humans did not occur to anyone. Early descriptions of the antagonistic actions of various microorganisms and molds were made 1874 by William Roberts and 1876 by John Tyndall [6]. In 1928, Alexander Fleming discovered that an agar plate containing staphylococci had been contaminated by a blue-green mold [7]. Around the mold was a halo of inhibited bacteria. He reasoned that some substances excreted by the mold must have inhibited the bacterial growth. The mold was later on identified as *Penicillium rubens* whereupon the substance was subsequently called penicillin [8]. His persistence and his belief in the idea made the difference to the scientists which had made similar observations before [9]. It took 12 years for him to get chemists interested in the extraction and purification of the substance,

which was finally published by an Oxford research group in 1940 [10]. Their protocol eventually led to penicillin mass production and distribution in 1945.

1.1.2 The Golden Era of Antibiotics

The three decades beginning with the 1940s are now called the golden era of antibiotics because in this time frame most of the antimicrobial substances still used today were discovered after the potential of microorganisms and fungi as sources for human drugs had been realized. Among the classes of antibiotics discovered in these era are the tetracyclines, macrolides, cephalosporines, aminoglycosides, chloramphenicols and rifamycins, one example of each is presented in Figure 1.1.

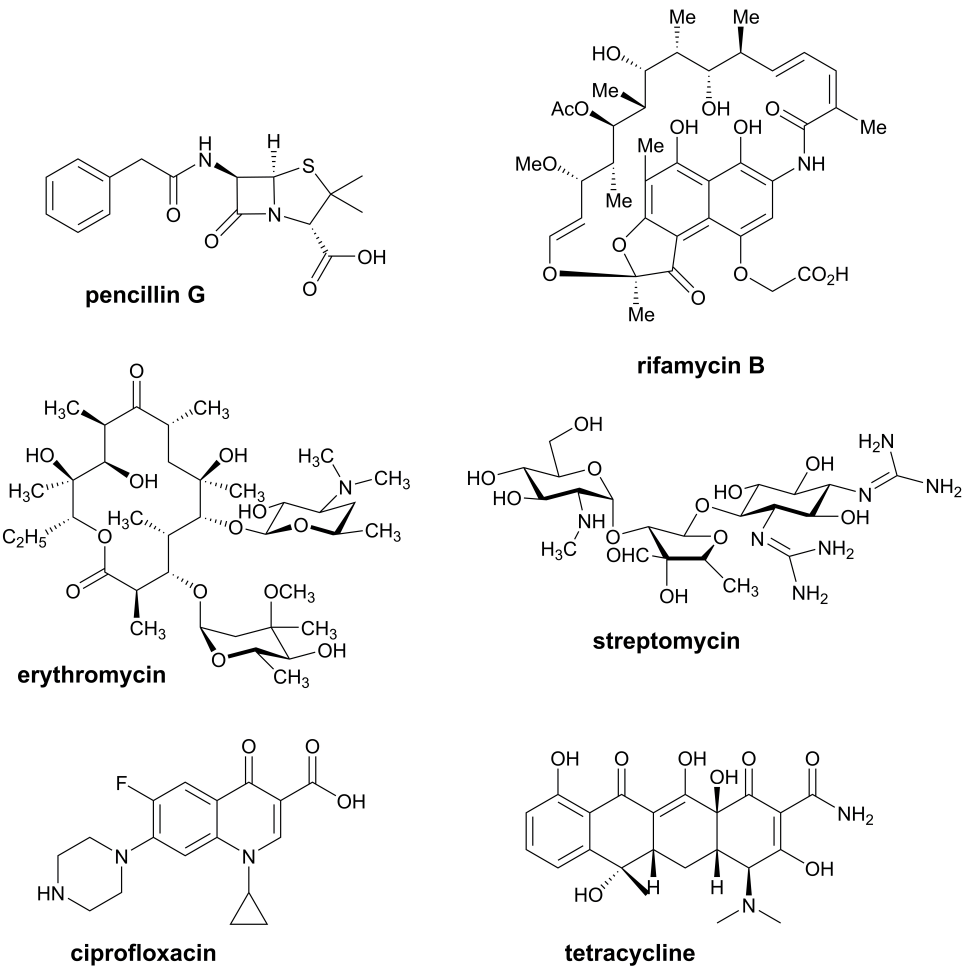

Figure 1.1: Chemical structures of representatives of the antibiotic classes discovered in the golden era of antibiotics. Pencillin G belongs to the class of β-lactams, erythromycin to the class of macrolides, streptomycin to the class of aminoglycosides and ciprofloxacin to the class of fluoroquinolones.

The discovery of streptomycin from the actinobacterium *Streptomyces griseus* in the 1940s prompted pharmaceutical companies to set up large research and development programs for natural product discovery. They consisted mainly of screening platforms for actinobacteria and fungi with activity against pathogenic bacteria. These organisms were isolated from soil samples collected all over the world. The testing for antibiotic activity was mainly done by phenotypic screening against different bacterial strains. This type of screening is still applied today as described in more detail in chapter 1.6.1. In the period from the 1940s to the 1970s, many of the important compounds still used today were isolated from *Streptomyces* strains, e.g. the macrolides, tetracyclines and aminoglycosides. With the exception of naladixic acid, an

NP-inspired synthetic derivative, all the antibacterial and antifungal drugs discovered in this era were natural products. In the 1970s, the focus of the screenings was broadened to anti-viral, anti-tumor and anti-parasitic activities as well as curative agents for non-infectious diseases [11, 12].
In the following years, the screening methodology stayed widely unchanged but target-based screening approaches were launched as well. Unfortunately, only a small number of natural products were discovered using this method, whereas numerous second, third or fourth generation semi- or completely synthetic derivatives of already known NPs were introduced to the market. During the 1990s, platforms for combinatorial chemistry were developed which presented the opportunity for the synthesis of thousands of new compounds. Advances in liquid handling technologies facilitated the high-throughput screening (HTS) of these compounds. This led to the fact that by the mid-1990s most pharmaceutical companies stopped screening natural products.

1.1.3 Natural Product Research Today

Despite the high contribution of natural products for the development of drugs, the interest in NP-based drug discovery has decreased drastically with the beginning of the 1980s. The reasons for this can be listed as the following [12, 13, 14]:

- After the "Convention of Biological Diversity" treaty was signed in the early 1990s, transfer and use of organisms from foreign countries has been hindered.
- When screening a microorganism library, surreptitious hits of already known compounds cannot be excluded.
- Natural products were seen as unfit for HTS screening because of non-specific interferences due to fluorescent or insoluble compounds in the crude extracts.
- Natural products are in some cases only produced in small amounts by the original producer, thus the availability of the substance is a problem.
- The high complexity of the molecules' structures results in chemical synthesis with low yields, if at all possible.
- It was believed that combinatorial synthesis techniques would suffice to provide new chemical lead structures.

The most promising way to overcome the faults of natural products was thought to be the combinatorial chemistry approach. However, until 2014 there was only one drug approved by the Food and Drug Administration (FDA) of the United States that is a formally a *de novo* synthetical chemical entity, namely the antitumor compound sorafenib (Nexavar) from Bayer [15]. In general, 31 % of the therapeutic agents approved between 1981 and 2014 were synthetic products (synthesized without combinatorial chemistry) whereas 46 % were natural products, derivatives thereof or NP-inspired synthetic products (Figure 1.2A). These numbers emphasize the important role

of natural products which to date cannot be met by synthetical molecules. This is mainly due to the fact that NPs are natural metabolites which implicates that they have general properties desirable for marketable drugs [16]. Thus, biologically relevant chemical space is better covered by natural products than by synthetic compounds as can be seen in Figure 1.2B.

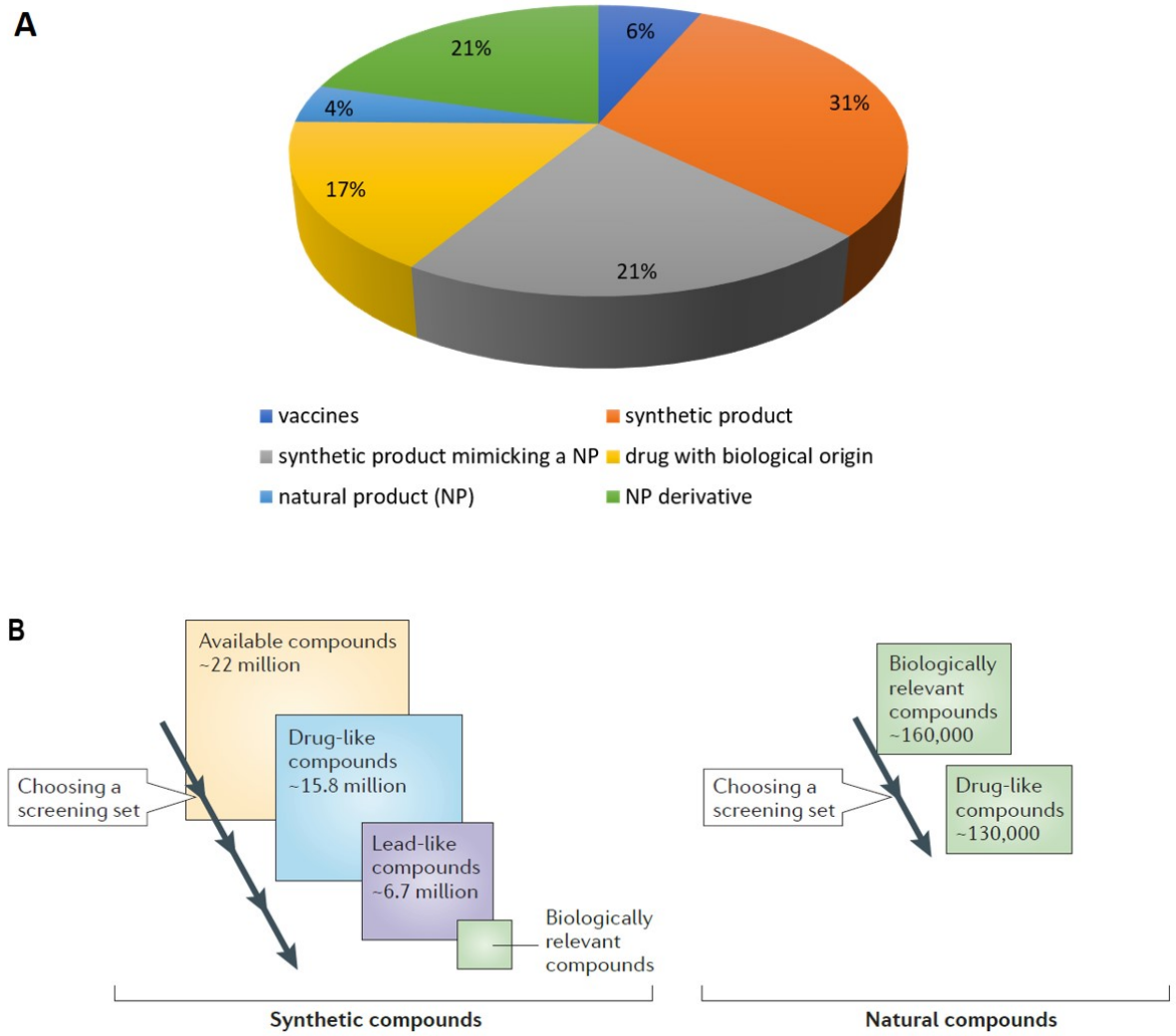

Figure 1.2: **A** Composition of all 1562 new drugs approved from 1981-2014 based on their origin (adapted from [17]). **B** The ZINC database harbors around 22 million commercially available synthetic products available for screening. As a consequence, considerable knowledge and effort is necessary to find biologically relevant compounds. In contrast, all 160,000 natural products listed in the Dictionary of Natural Products are biologically relevant of which 80 % are considered drug-like [13].

Consideration of the facts mentioned above led to an increase in natural product research in the last years which is accompanied by new efforts and

methods to increase its output, some of which are reviewed as the following:

- The connection between taxonomy and metabolic profile of an organism, known as chemotaxonomy, has come into focus in the past years. Accordingly, new natural products can be identified through metabolic profiling of an organism [18, 19].
- Fractionation of crude extracts to remove compounds that are likely to cause artefacts. Together with advances in detection technologies, most of the assays have become amenable to natural products [12].
- Using the "One Strain - Many Compounds" approach, the possibilities of production of various compounds under different cultivation conditions by one single strain are explored [20].
- Most natural products isolated so far derive from soil-originating actinobacteria or fungi (Table 1.2). The exploration of organisms from other habitats, like marine or endophytic environments is thought to provide promising sources for new chemical scaffolds [21].

Table 1.2: Approximate number of bioactive metabolites discovered in certain periods according to the producer organism (adapted from [22]).

Species	**1940-1974**	**1975-2000**	**2001-2010**	**Total**
Actinobacteria	3400	7200	3100	13700
Streptomyces sp.	2900	5100	2400	10400
Other actinobacteria	500	2100	700	3300
Microscopic bacteria	800	2300	1100	4200
Myxobacteriales	25	400	210	635
Cyanobacteria	10	30	1250	1290
Fungi	1300	7700	6600	15600

- As more and more whole genome sequences become available, natural product gene clusters can be explored *in silico*. Combinatorial biosynthesis, namely the mixing and matching of biosynthetic units like polyketide synthases (PKS), non-ribosomal peptide synthetases (NRPS) and glycosyl transferases, further leads to the creation of novel metabolites [13].

- Many secondary metabolites are only produced in small quantities by the original producer. Supply problems in the early stages of drug development should be addressed by small-scale parallel fermentation approaches as well as better metabolic understanding of these organisms [12].

1.2 Myxobacteria - A Proficient Source of Natural Products

Natural products deriving from microorganisms have been to date the most successful weapon against infectious diseases are a source for drug templates approved for the treatment of cancer and other civilatory diseases. Particularly, members of the order of Actinomycetales have provided over 3,000 antibiotics of which over 90 % are produced by the genus *Streptomyces* alone [23]. Most of the antibiotics on the market today, like streptomycin, chloramphenicol and tetracyclines are the original natural products or semisynthetic derivates thereof, produced by different *Streptomyces* species.
Another important contributor to antibiotic discovery has been the genus *Bacillus* with the production of lanthionine-containing antibiotics (lantibiotics), gramicidin and bacteriocin [24, 25, 26]. Among the eubacteria, there is a second group that produces a wide variety of chemical entities and over 450 antibiotics, called the myxobacteria [27]. Myxobacteria are δ-proteobacteria that live mainly in the soil, preferentially in places rich with organic matter and microbial life [28]. The order of Myxococcales consists of 55 species divided in 28 genera [29]. Due to their nutritional requirements, myxobacteria can be divided into two ecological groups that are also in agreement with their phylogeny. Predators, like those of the genus *Myxococcus*, use other bacteria or yeasts as food source. Cellulose decomposers, belonging mainly to the genus *Sorangium*, decompose organic materials [30].
Although myxobacteria have been frequently found in the soil, some species have also been isolated from a variety of environments all over the world [31, 32, 33], including fresh water [34] and sea water [35], moors [36, 37], anaerobic [38] and thermally extreme habitats [39, 40]. They exhibit a unique

social behaviour (Fig. 1.3): although they grow independently, they move by coordination of two different motility forms, the A- and S-motility [41]. Using this gliding process, they form swarms to prey for nutrients. By secreting exoenzymes and antibiotics, they are able to degrade organic matter or other bacteria and fungi [30, 42, 43]. If nutrients are scarce, the complex life cycle of myxobacteria presents an advantage in surviving. Under starvation conditions, the cells start to aggregate within the swarm, pile up and produce fruiting bodies. The shape of these fruiting bodies can vary from simple slime knobs containing myxospores in *Myxococcus* species to highly sophisticated designs resembling trees with branching slime stalks formed by *Chondromyces robustus* [28]. Within the fruiting bodies, vegetative cells transform into myxospores, an environmentally resistant resting state, that are firmly bound together. The myxospores can germinate and form a new swarm when the environmental conditions have improved [44, 45].

This social behaviour of myxobacteria throughout their lifetime, accurately related to as "moving, killing, feeding and surviving together" by Muñoz-Dorado *et al.* [45] rises the question how the cooperation of single cells is coordinated. Although the whole complexity of intercellular signalling has not been resolved, a number of studies have shed light on this particular field of research. It is known that *Myxococcus xanthus* produces large quantities of open membrane vesicles that contain hydrolytic enzymes and secondary metabolites [47, 48]. This kind of intercell signalling was also discovered to play a role in motility and fruiting body formation [49, 50]. The complexity of multicellular behaviour may also account for the fact that myxobacteria range among the prokaryotes with the largest genomes (13.0 Mb of *Sorangium cellulosum* So ce 56 [51]). Around 8 % of the genome of *Myxococcus xanthus* is dedicated to secondary metabolism. Because of their rich secondary metabolism, scientists eventually have discovered the myxobacteria as a proficient source for natural products particularly valuable as antibiotics, antifungals and cytotoxic agents.

The first account of antibacterial activity by a strain of *Myxococcus virescens*

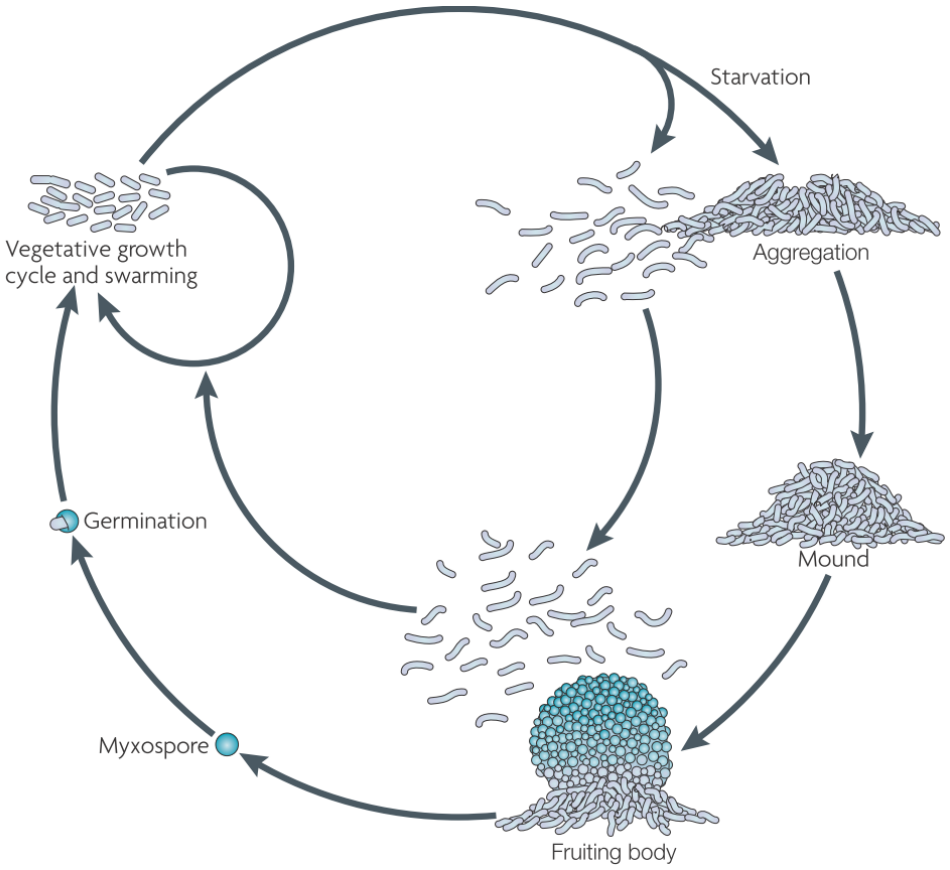

Figure 1.3: Life cycle of *Myxococcus xanthus*. When nutrients are present, cells divide and move in a swarm to find new resources. Upon starvation, cells aggregate to form mounds and macroscopic fruiting bodies that harbour the myxospores. When nutrients become available again, the spores germinate and complete the life cycle [46].

was described 1947 by Oxford [52]. The first isolation of a myxobacterial secondary metabolite, ambruticin, was reported in 1977 [53]. Since then, compounds with approximately 110 different core structures and hundreds of derivatives have been described [54]. Of these, around 54 % exhibit anti-fungal and anti-yeast activity [55]. A prominent example of anti-fungal myxobacterial products are the soraphens produced by *S. cellulosum* So ce26 (Fig. 1.4) [56]. The compounds are polyketide macrolactones, which incorporate an unusual, unsubstituted phenyl ring derived from benzoate [57]. Soraphen A, the most abundant derivative, exhibits strong activity against yeasts and molds, combined with only a moderate activity towards mouse fibroblasts. These encouraging results led to further steps in the development process towards utilization of soraphen as a plant-protective agent. Unfortunately, toxicological test revealed a teratogenic activity in mice which finally

led to the termination of the development project [58]. Nevertheless, the mode-of-action of soraphen A remains novel, as it inhibits acetyl-CoA carboxylase (ACC), a biotin-dependent enzyme that catalyzes the carboxylation of acetyl-CoA to produce the malonyl-CoA building block of fatty acids [59]. The biotin carboxylase domain of the ACC is blocked by soraphen which ultimately leads to a complete loss of its catalytic function [60].

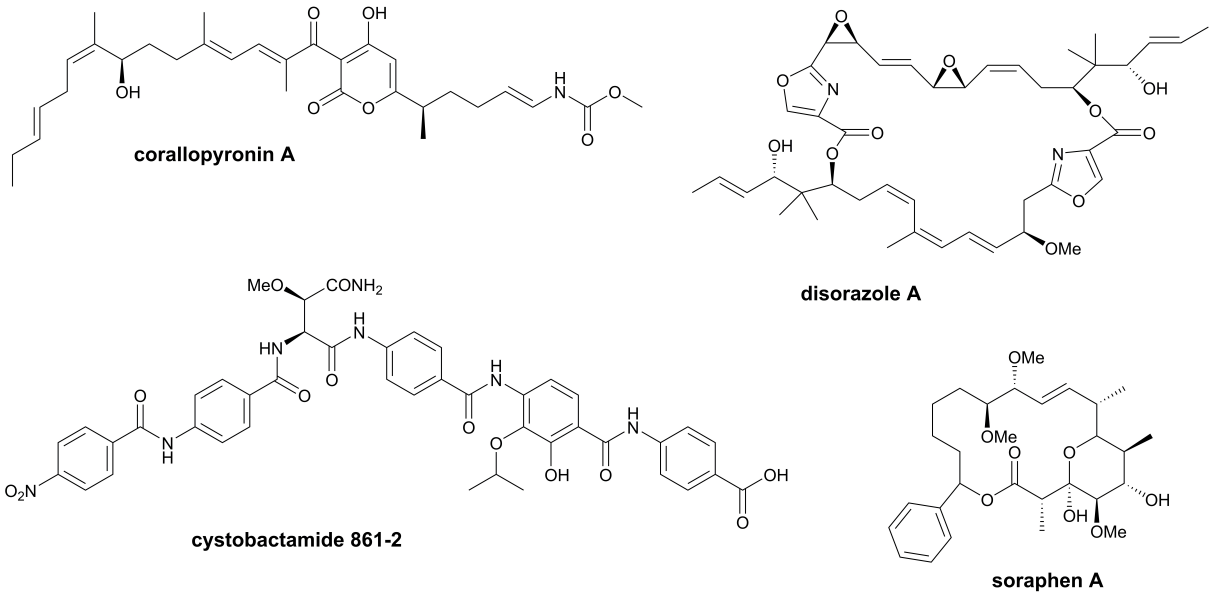

Figure 1.4: Chemical structures of natural products produced by myxobacteria.

Another *Sorangium cellulosum* strain, So ce12, is the producer of 26 different variants of the macrocyclic dilactone disorazol (Fig. 1.4) [61]. These compounds show very high cytotoxicity towards animal cells. Mode-of-action studies have demonstrated that disorazol A inhibits tubulin polymerization and leads to the depletion of microtubules [62, 63]. This perturbation to the microtubule network results in cell cycle arrest at the G_2/M checkpoint and induction of the apoptotic cell death cascade. Later on, the disorazoles have also been discovered to be active in anti-viral screens as they modulate important host functions and thus, interfere with virus entry, trafficking, and replication [64]. Recently, Rox *et al.* could show that the disorazols also block invasion of group A staphylococci into human epithelial cells, leading to efficient irradication of the pathogen [65]

Another high-profile compound class from myxobacteria are the cystobactamids produced by *Cystobacter* sp. Cb v34 [66]. The most potent derivative cystobactamid 861-2 exhibited activity in the low μg mL^{-1} range against a variety of Gram-positive and Gram-negative strains, thereby partly exceeding the activity of ciprofloxacin [67]. The bacterial gyrase could be identified as the molecular target of the cystobactamids. This enzyme changes the spatial orientation the double-stranded DNA molecules prior to replication or transcription. As the cystobactamids have been discovered fairly recently, they are currently being subjected to extensive tests for the evaluation of their full potential.

1.2.1 The Myxopyronins

In 2015, 1.8 million people died of tuberculosis, according to the World Health Organization (WHO). This fact makes the infectious disease caused by *Mycobacterium tuberculosis* one of the top 10 causes of deaths worldwide [68]. Due to the extensive treatment of tuberculosis using a combination of different antibiotics, first cases of resistance of *M. tuberculosis* to rifampicin, the antibiotic of choice, were reported in 1993 [69]. In 2015, 3.9 % of new tuberculosis cases and 21 % of previously treated ones involved strains with rifampicin or rifampicin/isoniazid resistance [68]. Consequently, there is an urgent need for new antibacterial agents for the treatment of this wide-spread disease. Among the promising substances are the myxopyronins, α-pyrone natural antibiotics (Figure 1.5) produced by the gram-negative myxobacterium *Myxococcus fulvus* Mx f50 [70].

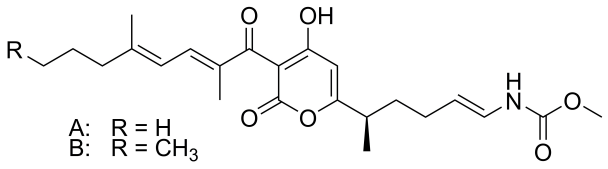

Figure 1.5: Chemical structure of the myxopyronins.

They target the switch region of the bacterial DNA-dependent RNA-polymerase (RNAP) which is a different site than the one targeted by the

rifamycins, therefore avoiding cross-resistances (Fig. 1.6) [71, 72, 73].

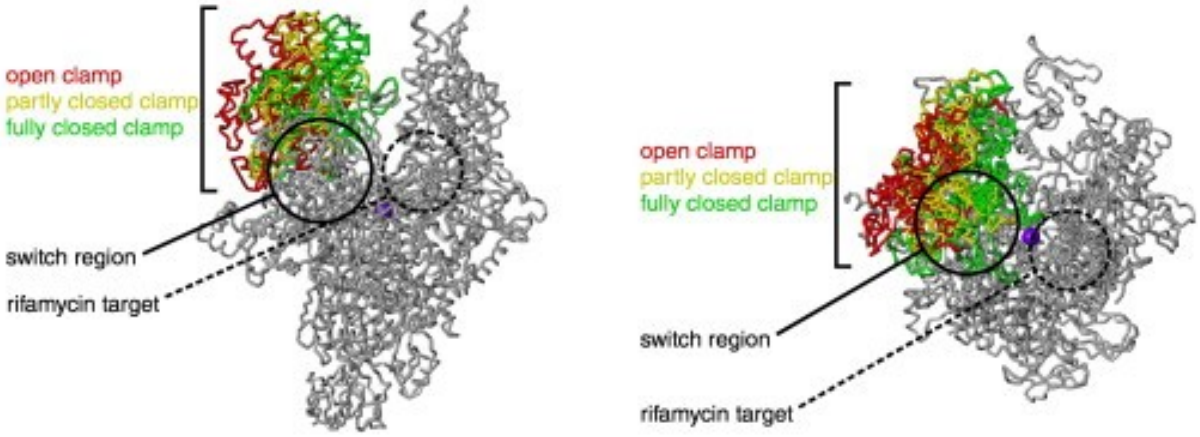

Figure 1.6: Conformational stages of the RNAP showing the target sites for rifamycin and the switch region targeted by myxopyronin [71].

Although there is no cross-resistance to RNAP inhibitors currently on the market the frequency of resistance was calculated to be in the same range as this of rifampicin [72]. Furthermore, mutations of the target region were found that were induced by myxopyronin [74, 75]. This makes myxopyronin in its native structure an undesirable candidate as an antibacterial agent. However, myxopyronin is used as a model PKS-derived compound for evaluation of the potential of heterologous expression of myxobacterial products in *Myxococcus xanthus.*

1.2.2 The Vioprolides

Vioprolides are cyclic peptolides produced by the myxobacterium *Cystobacter violaceus*, recently classified as *Archangium violaceum*, Cb vi35 [76, 77]. They are remarkable examples for the structural diversity displayed by myxobacterial natural products, as they are one of the very few compounds containing an azetidine ring (Fig. 1.7). Furthermore, the structures contain unusual amino acids. In vioprolide A (1) and B (2), one proline is replaced by a pipecolic acid. In vioprolide A (1) and C (3), another proline is replaced by a 4-methyl azetidine carboxylic acid (4-Maz).

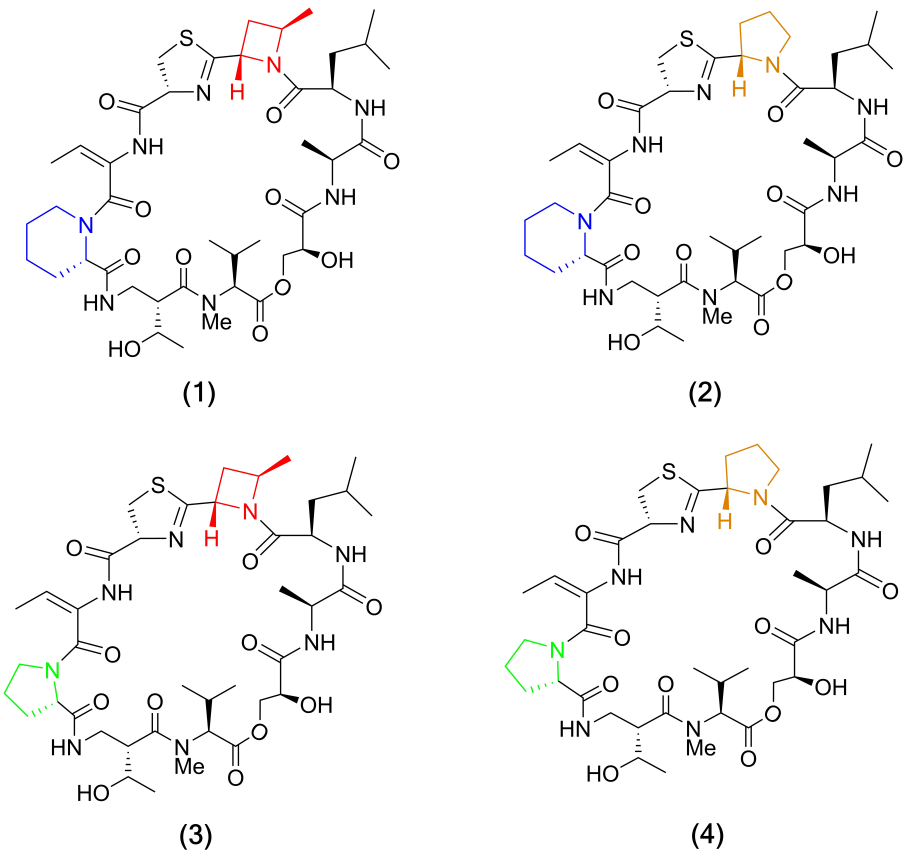

Figure 1.7: Chemical structure of the vioprolides. (1) vioprolide A, (2) vioprolide B, (3) vioprolide C and (4) vioprolide D. The four derivatives are distinguished by the incorporation of either L-pipecolic acid (blue) or L-proline (green), as well as either 4-methylazetidinecarboxylic acid (red) or L-proline (orange).

The vioprolides exert antifungal as well as cytotoxic and immunomodulatory activity [77, 78, 79]. Vioprolide D (4) shows the highest activity against a variety of fungi and yeast with the least cytotoxicity against mammalian cells, whereas vioprolides A-C (1-3) show a higher cytotoxicity and a lower antifungal activity. The immunomodulatory activity is based on the ability of these compounds to modulate the interferon response, involved in all kinds of inflammatory or autoimmune diseases [78, 79]. The antifungal activity of vioprolide D may also be even more important, since two of the three classes of antifungal drugs used today (azoles and polyenes) have already been launched in the 1980s with the third group, the echinocandins, having been discovered at that time as well [80]. In contrast, the increased incidence rate of invasive fungal infections that are in need of treatment can be correlated with an expansion of the number of permanently immunocompromised

patients, like those treated because of AIDS/HIV, cancer therapy or organ transplantation [81]. Furthermore, strains of *Candida albicans* or *Candida glabrata* with resistances to currently used drugs are emerging as well [82]. In this context, vioprolide D is a promising candidate for the treatment of invasive fungal infections, as it combines antifungal and immunomodulatory activity. This is noteworthy, because it has been shown that type I interferon-signaling mediates the lethal hyper-inflammatory response during systemic infections of *Candida albicans* in the mouse model [83].

1.3 Natural Products Produced by the Genera *Chitinophaga* and *Flexibacter*

The genus *Chitinophaga* was proposed by Sangkhobol & Skerman in 1981 to include strains of filamentous, chitinolytic gliding bacteria that form spherical rods upon aging [84]. The genus was assigned to the order of Sphingobacteriales in the phylum Bacteriodetes. Soon after their identification, similarities between *Sporophytophaga*, *Flexibacter* and *Chitinophaga* strains [85, 86] were pointed out which finally led to the re-assignment of several *Flexibacter* and one *Cytophaga* strain to the genus *Chitinophaga* [87]. This genus now comprises a number of heterogenous strains with regards to gliding motility, habitat and chitinolytic activity. As the pyhlum of Bacteroidetes is a subject to change and especially the genus *Flexibacter* is proposed to be subdivided in to different genera [88], the potential for the production of natural products of these two genera is reviewed together.

Several β-lactam antibiotics with interesting modes-of-action were isolated from *Flexibacter* strains (Fig.1.8). The formadicins from *Flexibacter alginoliquefaciens* act selectively against pseudomonads and have proven hydrolysis-resistant against various types of β-lactamases [89, 90]. The monobactam SQ 28332 94 also belongs to the group of β-lactams from the genus *Flexibacter* [91]. The dipeptides TAN-1057A-D produced by a *Flexibacter* strain [92, 93] were shown to inhibit peptide elongation during bacte-

rial translation due to inhibition of the peptidyl transferase reaction, thereby possessing a different binding site than other translational inhibitors [94].

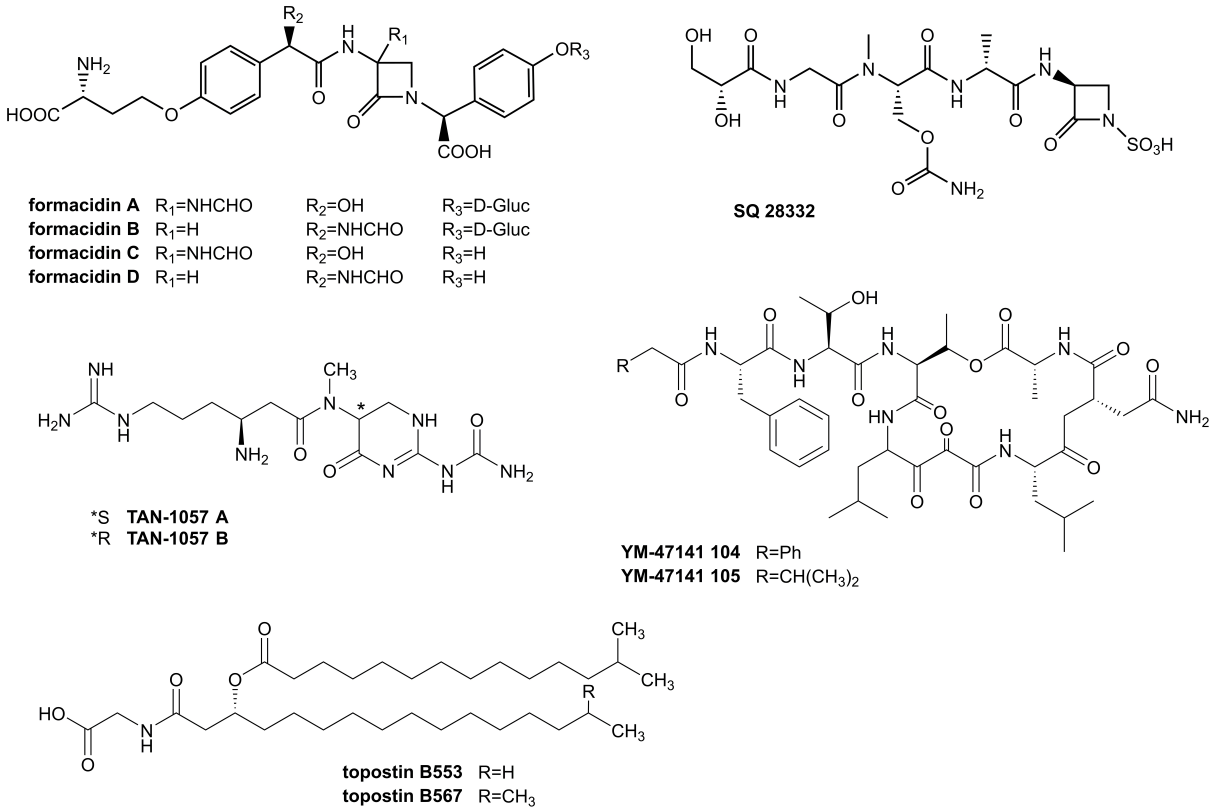

Figure 1.8: Chemical structures of natural products produced by the genus *Flexibacter*.

Besides antibiotics, proteinase inhibitors were also found to be produced by several *Flexibacter* strains, such as the human leukocyte elastase inhibitor FR901451 103 [95, 96]. Two other elastase inhibitors, designated YM-47141 104 and YM-74142 105 are cyclic depsipeptides displaying a tricarbonyl moiety which is suggested to be essential for their function [97, 98]. Compounds isolated from *Flexibacter topostinus* were identified as lipid-containing amino acids and peptides which were shown to suppress topoisomerase I activity and were subsequently called topostins [99, 100, 101].

Chitinophaga strains have been isolated from carbon-rich soil samples around the world [102, 103]. To date, only two groups of natural products have been isolated from this genus, namely the pinensins from *Chitinophaga pinensis* [104] and the elansolids from *Chitinophaga sancti* [105] (Fig. 1.9). But since Wyatt *et al.* have found not yet annotated biosynthetic gene clusters in

other *Chitinophaga* strains, the potential of this genus may have not been fully discovered yet [106].

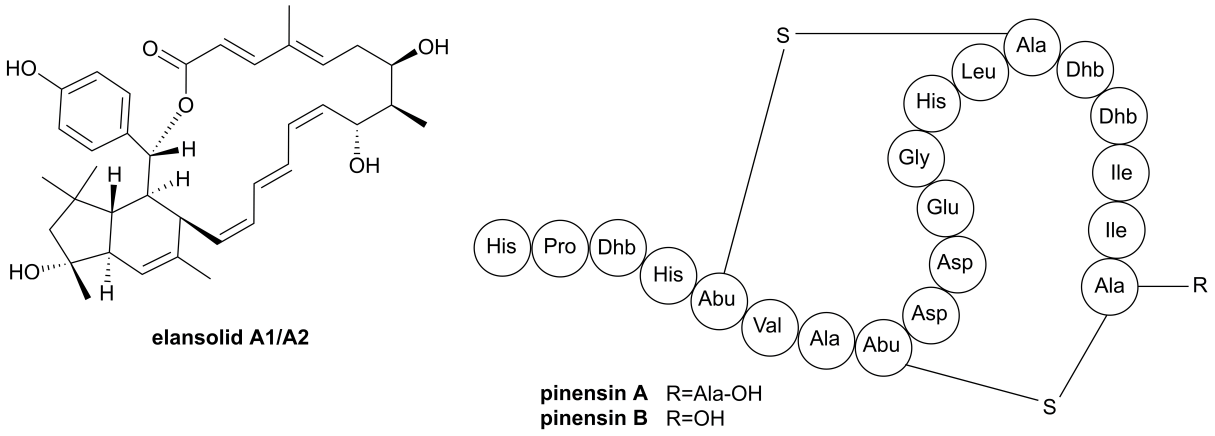

Figure 1.9: Chemical structures of natural products produced by the genus *Chitinophaga*.

The pinensins are lantibiotics that were found to be highly active against many filamentous fungi and yeast but showed only a weak bacteriocidal activity [104]. This is unusual, as lantibiotics are mostly produced by Gram-negative bacteria and had thus far been found to act only against Gram-negative bacteria [107]. Therefore, pinensins are regarded as the first antifungal lantibiotics.

1.3.1 The Elansolids

During the search for novel antibiotics, Steinmetz *et al.* found a group of new macrolides, for which they proposed the trivial names, elansolids (Figure 1.10), in the culture extracts of the Gram-negative soil-dwelling bacterium *Chitinophaga sancti* [105, 108].

Some of the elansolids exhibited moderate to strong activity against several Gram-positive bacterial pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA is at present the most commonly identified antibiotic-resistant pathogen in many parts of the world with a prevalence between 25 % and 50 % in most parts of America, Australia and southern Europe [109, 110]. In 2005, 19,000 deaths associated with MRSA strains were reported in the USA [111]. Although infection rates are decreasing, MRSA

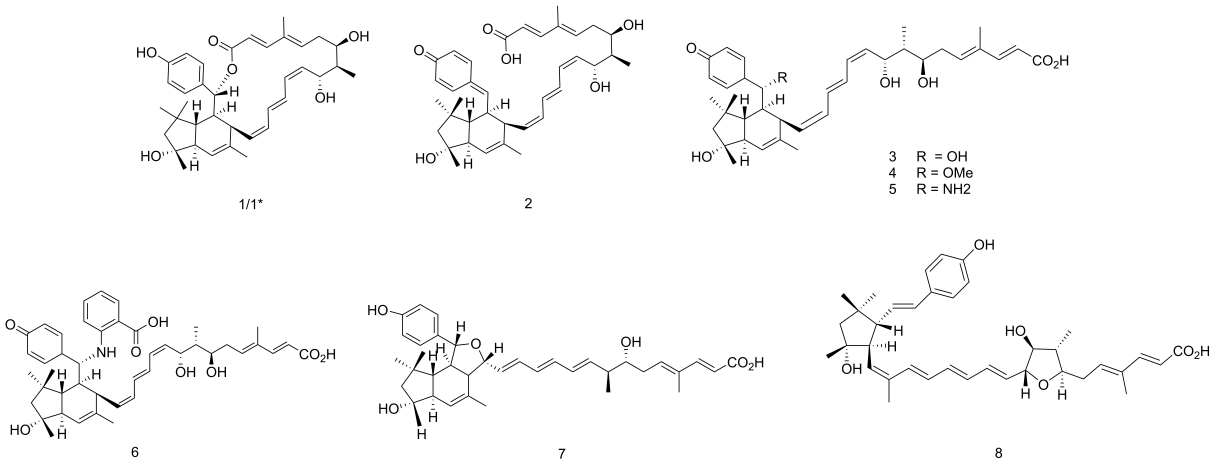

Figure 1.10: Chemical structures of elansolids. 1/1* = elansolid A1/A2, 2 = elansolid A3, 3 = elansolid B1, 4 = elansolid B2, 5 = elansolid B3, 6 = elansolid C1, 7 = elansolid D1, 8 = elansolid D2.

infections were estimated to affect more than 150,000 patients in the EU alone in 2010 [112]. Until recently, vancomycin and daptomycin have been the only effective treatment for methicillin-resistant pathogens [113]. But in 1997, the first vancomycin intermediate-resistant *S. aureus* strains were isolated, followed by reports of vancomycin resistant ones in 2003 [114, 115]. Cases of daptomycin-resistant MRSA have also been described [116, 117]. Consequently, the need for new antibiotics for the treatment of these multi-resistant bacteria arises. In a global priority list released by the WHO in 2017, development of antibiotics against MRSA is given the second highest priority [118]. To tackle this threat, efforts are undertaken to find new substances which can be developed as antibacterials.

Extensive chemical and biological studies revealed that elansolids A1 and A2 (1/1*) occur as two distinct atropisomers. Both elansolids exhibit potent activity against Gram-positive bacteria, elansolid A2 being generally more active than elansolid A1. The MIC (minimal inhibiting concentration) of elansolid A2 against methicillin-resistant *S. aureus* strain MRS3 is in the range of 2 μg mL^{-1} [17]. Further studies revealed that the final product of the biosynthetic pathway is deoxyelansolid A3 (2) which undergoes lactonization by an intramolecular Michael addition to yield elansolid A1/A2 [108, 119].

Water, methanol or ammonia can also serve as nucleophiles that attack the quinone methide moiety of elansolid A3 to generate elansolids B1-B3 (3-5) and D1-D2 (7-8) [119] (Figure 1.11).

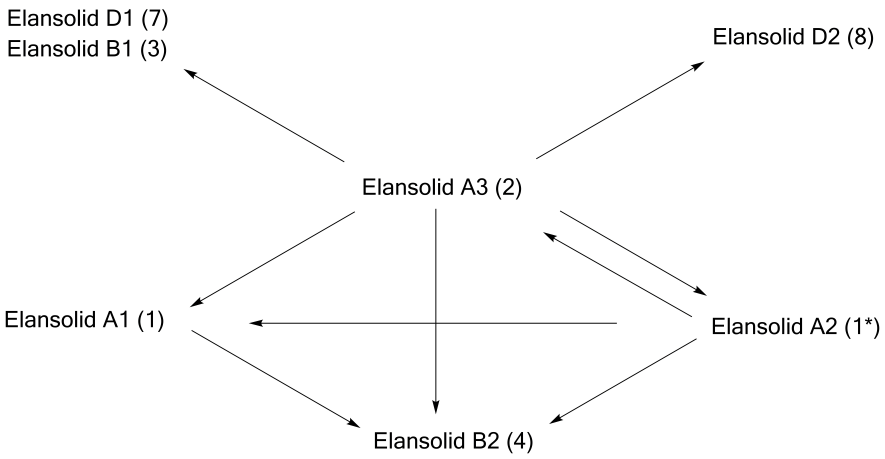

Figure 1.11: Interconversion of elansolids (adapted from [119]).

This reaction can be exploited in two different ways: By adding different precursors as nucleophiles to the crude extracts of a *C. sancti* culture to create new elansolid derivatives in a precursor-directed synthesis or by feeding precursors such as anthranilic acid directly to the culture medium for a directed biosynthesis (Figure 1.12). Interestingly, the bioactivities of these newly formed derivatives were roughly in the same range as that of elansolid A2 [120]. These results are promising as they possibly allow the creation of precisely tailored derivatives with increased efficacy, once mode of action studies have revealed the target of the elansolids.

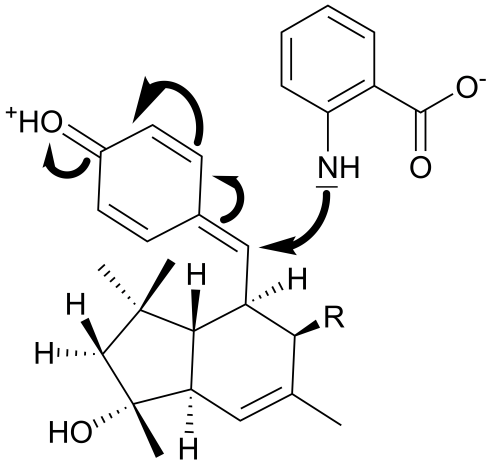

Figure 1.12: Reaction mechanism for the formation of elansolid C1 (6) by a Michael-type nucleophilic attack of anthranilic acid on the *p*-quinone moiety of elansolid A3 (2). Anthranilic acid can be substituted by other nucleophiles to yield the corresponding derivative.

1.4 Natural Product Biosynthesis

Most of the natural products relevant in clinical use today originate from the secondary metabolism of the producer strain. They exhibit an extraordinary chemical diversity which leads to a wide range of pharmaceutical functions like antibiotics, immunosuppressants, antiparasitics, hypolipidemics and anti-tumoral agents [121]. In the majority of cases, the bioactive compounds are the end products of complex multi-step biosynthetic processes. Terpenes are the largest class of small-molecule natural products on earth, and the most abundant by mass. They are built by plants, some insects and fungi and are synthesized from dimethylallyl diphosphate and isopentenyl diphosphate by different prenyl transferases and terpene synthases [122]. Besides the terpenes, there are giant multifunctional enzyme systems called polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) that assemble secondary metabolites mostly from simple building blocks such as coenzyme-A-activated carboxylic acids and amino acids [123]. Although they utilize different chemistries for activation and condensation of their substrates, PKSs and NRPSs show striking similarities in their buildup. This also leads to the fact that there are also PKS-NRPS hybrid systems.

1.4.1 Polyketide Biosynthesis

The different types of PKS together with their building blocks and prominent examples are listed in Table 1.3.

Table 1.3: List of the different types of PKSs (adapted from [124]).

PKS type	**Building Block**	**Organism**	**Example**
Modular type I (non-iterative)	ACP, various extender units (*in situ* methylation possible)	Bacteria, protists	Erythromycin [125]
Iterative type I	ACP, only malonyl-CoA extenders (*in situ* methylation possible)	Mainly fungi, some bacteria	Lovastatin [126]
(Iterative) type II	ACP, only malonyl-CoA extenders	Exclusively bacteria	Doxorubicin [127]
(Iterative) type III	Acyl-CoA, only malonyl extenders	Mainly plants, some bacteria and fungi	Flavolin [128]
PKS-NRPS hybrid	ACP, malonyl-CoA, amino acids	Bacteria (modular), fungi (iterative)	Epothilone [129]

Most of the natural products built by mxobacteria and other bacteria, like these of the genus *Chitinophaga*, are synthesized by modular non-iterative type I PKS (for exceptions of myxobacterial products, see reference [130]). The biosynthesis of those polyketides resembles the formation of fatty acids. The reactions are carried out by linearly arranged, covalently fused catalytic domains paired up into functional modules. Each module contains a minimum of three domains required to catalyze one cycle of chain extension: Ketosynthase (KS), acyltransferase (AT) and acyl carrier protein (ACP) as well as a variable set of domains associated with keto group modification (ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER)) [131, 132]. Each domain is presumed to form a localized globular structure with a catalytic role determined by conserved residues at its specific active site. The domains are attached to each other by linker regions consisting of up to 100 amino acids. The number of modules usually correlates with the number of

extension cycles executed by the PKS (principle of collinearity) [124]. The presence of KR, DH and ER domains defines the degree of β-keto processing. The gene sequences for the enzymes required to build one molecule are usually in close promiximity to each other and are called biosynthetic gene cluster. As the function of an enzyme is largely determined by its residues at the active site, a genome sequence can be searched for these sequences in order to identify novel biosynthetic gene clusters [133]. Sometimes, the module architecture does not fit with the resulting metabolite structure. This can be due to the fact that at times modules are skipped or used more than once, like in the case of stigmatellin [134, 135]. Another reason can be the occurrence of PKS-subclass called trans-AT PKS. Here, the modules lack individual AT domains. Instead, the ACPs are loaded by stand-alone ATPs that are encoded on individual genes [136, 137].

In Figure 1.13 a schematic visualization of four modules of a type I PKS is presented. The first domain of the loading module (LM), namely the acyl carrier protein (ACP), is usually loaded with one acetyl-CoA moiety to start the biosynthesis. In some cases also bigger starting units can be used, like propionyl-CoA for myxopyronin B [138]. Every ACP is naturally an apoenzyme (inactive state) and must be activated by transferring 4-phosphopantetheine from co-enzyme A to a conserved serine of the ACP, resulting in the holoenzyme (active state) [132]. This reaction is carried out by a phosphopantetheinyl transferase. The ACPs of the following modules are loaded with malonyl-extender units (originating from malonyl-CoA) with the help of the AT. Instead of malonate, also methyl malonate, ethyl malonate or other extender units can be used [139]. In trans-AT PKS systems, the same trans-ATs are used iteratively to acylate each module which leads to the consecutive incorporation of the same type of building block. In these PKSs, methyl branches are therefore usually inserted by methyl transferases (MTs) [136].

The growing chain is transferred from the ACP to the ketosynthase (KS). This enzyme catalyzes a Claisen thioester condensation under carboxylation

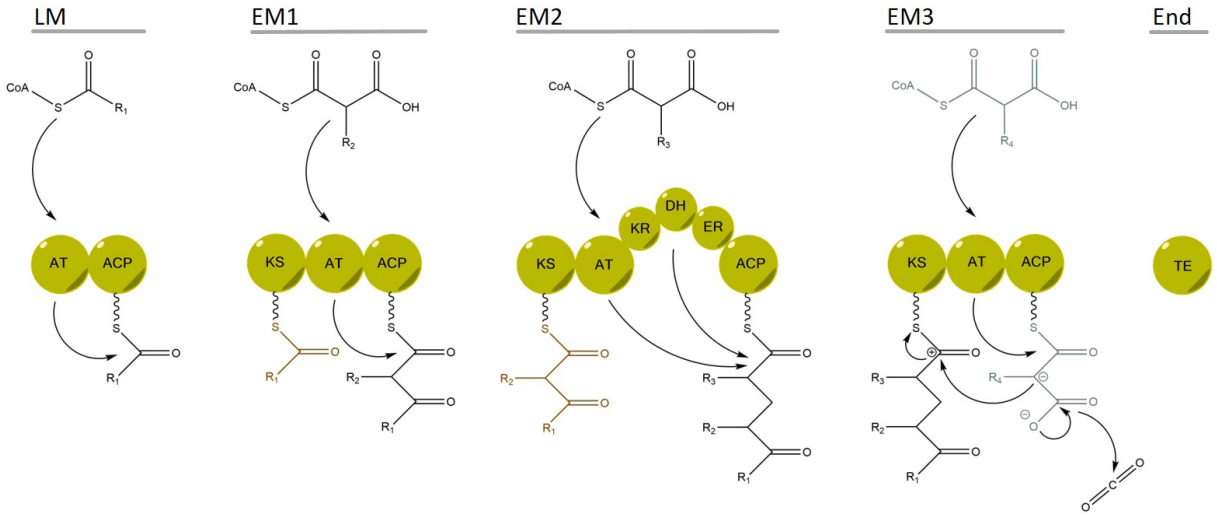

Figure 1.13: Schematic visualization of PKS biosynthesis. The ocher bubbles illustrate the individual enzymatic domains which are organized in modules. The extended polyketide chain is represented in brown, the extender units in black. EM3 shows the mechanism of chain extension.

of C_3 of the extender unit [121]. The C_2 of the extender units initiates a nucleophilic attack at the C_1 of the chain bound to the KS. The elongated chain is transferred to the ACP afterwards. EM1 (extender module 1) shows the chain after the first elongation step. C_3 with the attached rest R_1 derives from the acteyl starting unit, whereas C_1 and C_2 with the attached rest R_2 derive from the extender unit. EM1 is called a minimal extension unit because it consists only of a KS, AT and ACP module. EM2 on the other hand is called a complete reductive loop because it additionally posses a KR, DH and ER [140]. After chain elongation, the KR reduces the β-keto group to an alcohol. The DH reduces the product again under elimination of water to form a double bond (with *cis*- or *trans*-configuration). Afterwards, the ER carries out another reduction to form a single bond and with it a fully saturated acyl backbone. When the required chain length is reached, the chain is transferred to a thioesterase (TE) which releases it. At release often an intramolecular cylcization occurs [141].

The products emerging of such a PKS pathway can be very complex because with every step two stereo centers can be added. Additionally, conserved protein cassettes have been discovered that catalyze the addition of methyl

groups to the electrophilic β-carbon, a process called β-branching [139]. The diversity can also be increased by post-PKS steps such as C-, O- and N-glycolyzations, alkylations, acyl transfers, hydroxylations end epoxydations. Other known modifications involve halogenation, transamination, nitrile formation and desaturation to yield alkynes [142].

1.4.2 Non-Ribosomal Peptide Synthesis

The buildup of the Non-Ribosomal Peptides Synthetase (NRPS) resembles this of the PKS because of its modular structure. The modules can also be dissected into catalytic domains each being responsible for a specific step during peptide synthesis. One elongation module contains at least three essential domains: A condensation domain (C), an adenylation domain (A) and a peptidyl carrier protein (PCP) (Figure 1.14A). An initiation module comprises only the A-PCP domains.

The A-domain is responsible for the selection of the amino acids that make up the product and thus controls the primary sequence of the peptide. The specificity for a certain amino acid is encoded by up to eight amino acid residues in the substrate binding pocket of the A-domain. Besides proteinogenic amino acids, a plethora of non-proteinogenic amino acids is found to be integrated which increases the structural diversity of the final products [143]. A-domains activate amino acid substrates as amino acyl adenylate through ATP hydrolysis [144]. Before this reactive intermediate can be further transported to the PCP-domain, the latter has to be activated by transferral of the co-factor 4-phosphopantetheine (PPan) onto a conserved serine residue of the PCP. The reactive amino acyl adenylate is then transported onto the thiol moiety of the PPan. The holo-PCP is responsible for the transport of the thioester bound substrates and all elongation intermediates between the catalytic active sites and is therefore referred to as the 'swinging arm' of each NRPS module [145, 143]. In the next step of NRP-synthesis, the C-domain catalyzes the peptide bond formation between amino acyl substrates bound to PCPs of adjacent modules. The peptide bond formation is initiated by a

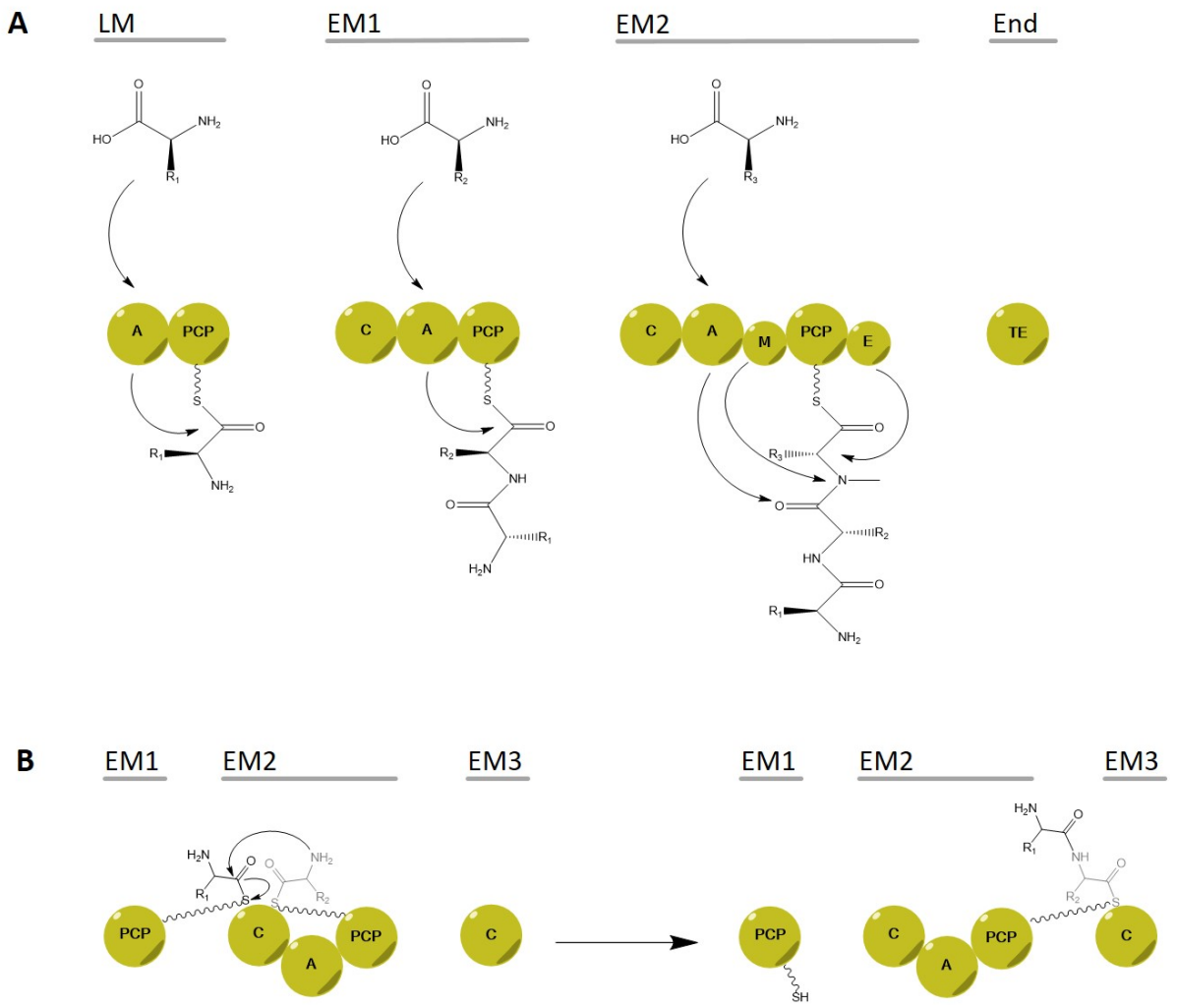

Figure 1.14: **A** Schematic visualization of NRPS biosynthesis. The ocher bubbles illustrate the individual enzymatic domains which are organized in modules. **B** shows the mechanism of chain extension.

nucleophilic attack of the amino group of the activated amino acid bound to the downstream (with respect to the C-domain) module onto the acyl group of the amino acid tethered to the upstream module (Fig. 1.14B) [144]. The resulting peptidyl intermediate is then translocated down the assembly line for chain elongation and further modification steps. These tailoring enzymes fall into two categories: Some are integral parts of the NRPS and act in *cis*, whereas others are distinct enzymes acting in *trans*.

A range of tailoring domains that are integrated into the elongation modules have been discovered. The most prominent ones are epimerization (E), methylation (M), cyclization (Cy), oxidation (Ox), reduction (R) and formy-

lation (F) domains [146]. E-domains catalyze the generation of D-amino acids from their corresponding L-isomers. M-domains on the other hand use the co-factor S-adenosyl-methionine for N- and C-methylation of specific amino acid residues, thus making the peptide less susceptible to proteolytic breakdown [144]. In some NRPS modules that select cysteine, serine or threonine residues, integrated Cy-domains catalyze a heterocylcic ring formation to the corresponding thiazoline or oxaxoline rings in addition to peptide bond formation. The heterocycles formed by the Cy-domains can be further catalyzed to thiazole or oxazole rings by Ox-domains, whereas R-domains catalyze the formation of thiazolidine or oxazolidine rings [145]. In some NP assembly lines, like those for gramicidin, a F-domain is attached to the initiation module that catalyzes the formylation of the first amino acid [147]. In addition to the modifications that occur during NRPS assembly by the integrated tailoring domains, other modifications can also take place by independent enzymes that temporarily associate with the NRPS template. Vancomycin, for example, is cross-linked by oxidative cyclization of the phenolic side chain by independet cytochrome P450-type heme proteins [148]. Afterwards, the aglycon scaffold is decorated with sugar residues by an independent glycosyltransferase [149]. In many glyosylated NRPs, the sugars have a major impact on their bioactivity [150].
In parallel to the PKS system, there are also examples where NRPS modules are used iteratively, which often leads to a symmetry of the product. A further variation of the iterative mechanism is the nonlinear mode, which relies on the fact that only one domain is used more than once during NRP biosynthesis [143].

1.5 Heterologous Expression of Natural Products

One major drawback of myxobacterial compounds on their way to being developed as marketable products are the low product titers obtained from the original producers. Total synthesis has already been achieved for some products [151, 152, 153] but remains daunting because of the variety of functional

groups and multiple stereocenters that are characteristic to natural products, resulting in low overall yields. Another approach to overcome this obstacle is the expression of myxobacterial compounds in a heterologous host with the aim of improved product concentrations. The ideal host should have the following features [154, 155, 156, 157]:

- a relatively fast growth rate
- be suitable for bioreactor fermentations
- be amenable for genetic modifications
- the genome should be fully sequenced
- recognize all elements of the gene cluster to ensure functional expression
- have a broad precursor supply for NRPs and PKs (methylmalonyl-CoA, propionyl-CoA, non-proteinogenic amino acids)
- have a similar GC-content resulting in a similar codon usage
- possess a broad range of 4'-phosphopantetheinyl transferases (PPTases) for activation of the biosynthetic machinery
- have a minimal secondary metabolite background to lower the general metabolic burden
- knowledge of gene cluster regulation, pathway interactions and metabolic fluxes should also be taken into account

The first four requirements are met by the model organisms *Streptomyces coelicolor, Streptomyces lividans, Escherichia coli* and *Pseudomonas putida*, whereas the other favorable features are often met by strains that are phylogenetically closely related, but have better growth and genetic characteristics than the original producers.
As genetic modifications of myxobacteria pose a challenge, especially since no plasmids have been isolated so far, first heterologous expression approaches for the breast cancer lead compound epothilon from *Sorangium cellulosum*

SMP44 were conducted with *S. coelicolor* CH999 [158]. *S. coelicolor* has a doubling time of 2 h versus 16 h for *S. cellulosum* and is genetically well understood. Because of its capability of endogenous polyketide production, e.g. of actinorhodin, it was thought to be a suitable candidate. Nevertheless, only small quantities (50-100 μg L^{-1}) were produced, possibly because of cytotoxic effects of epothilone on the host strain [158].

Production of soraphen A could be achieved in *Streptomyces lividans* after augmentation of the host with a methoxymalonate-CoA and a benzoyl-CoA biosynthetic pathway in addition to the soraphen gene cluster [159]. Here, the yield was 300 μg L^{-1} and therefore six times lower than the initial titers in the original producer *Sorangium cellulosum* So ce26 [56].

The use of *E. coli*, normally the first choice for heterologous expression of prokaryotic molecules, takes enormous effort because firstly the GC content and therefore the codon usage differ significantly to myxobacteria which can lead to a non-efficient expression and secondly *E. coli* lacks PPTases that interact with modular PKS proteins [160]. These enzymes catalyze the covalent attachment of 4'-phosphopantetheinate derived from coenzyme A to the peptidyl (for NRPS) or acyl (for PKS) carrier proteins, converting these enzymes from the inactive apo- to the active holo-form. The endogenous PPTases of *E. coli* are not able to carry out this reaction. Thirdly, the organism is not able to provide the necessary precursors for PK and NRP synthesis. The latter issues have been addressed by introducing the SFP PPTase from *Bacillus subtilis* into the genome of *E. coli* and by engineering the host strain to supply propionyl-CoA and methylmalonyl-CoA, PK precursors that are uncommon in *E. coli* [161, 162]. By using this strain and by optimization of codon usage, lowering of the cultivation temperature, co-expression of chaperones and splitting the largest protein into two polypeptides, Mutka *et al.* were able to produce epothilone C and D, however only in concentrations of about 10 μg L^{-1} [163].

Most of the undesirable characteristics of *E. coli* are circumvented when utilizing *Pseudomonas putida* as the expression host. The advantages of

P. putida are the fully sequenced genomes of important strains like KT2440 accompanied by numerous tools for genetic manipulation and gene expression as well as its high GC content similar to this of myxobacteria. Other desirable features are a high tolerance towards xenobiotics due to its effective efflux system, a rather 'clean' metabolic background and the ability to produce the precursors acetyl-CoA, propionyl-CoA and malonyl-CoA [164]. The major advantage however is the provision of a broad substrate range with the ability to activate ACP and PCP domains [165]. The research group of Rolf Müller has successfully expressed several myxobacterial products in *P. putida* KT2440. The first product was myxochromide S from *Stigmatella aurantiaca* DW4/3-1 with a product titer of 40 mg L^{-1} compared to 8 mg L^{-1} in the original host [135, 166]. Unfortunately, the product was not exported to the medium which poses a disadvantage for later purification steps. Another example is the expression of the PKS/NRPS hybrid gene cluster of myxothiazol A also originating from *Stigmatella aurantiaca* DW4/3-1 [167]. After engineering the host strain into providing the necessary precursor methlymalonyl-CoA, myxothiazol A concentration was 600 μg L^{-1} [168, 169]. The production of pretubulysin, originally from *Cystobacter* sp. SBCb004, was achieved with a titer of 1.76 μg L^{-1} through exchange of the rare start codon TTG and feeding of pipecolic acid [170, 171].

Reviewing these efforts for heterologous expression of myxobacterial compounds it becomes obvious that the results are far from satisfactory due to the problems accompanying expression in non-related hosts. Another option for achieving higher product titers is the expression in a phylogenetically related strain with better growth characteristics. *Myxococcus xanthus* is to date the best characterized myxobacterium with a fully sequenced genome that possesses a considerably shorter doubling time with 5 h than e.g. *Sorangium cellulosum* with 16 h [51, 172]. Furthermore, there is no engineering of promoters necessary and biosynthetic precursors as well as PPTase are intrinsically available. This is why *M. xanthus* was used in a number of studies for the heterologous expression of myxobacterial compounds. After

epothilone production in *Streptomyces coelicolor* yielded only small quantities, Julien & Shah reconstituted the gene cluster into the chromosome of *M. xanthus* via homologous recombination. At first, product concentrations were also quite low, in the range of 500 μg L^{-1}, but with media optimization and a feeding strategy using casitone and methyl oleate, titers of 23 mg L^{-1} epothilone could be achieved, which is comparable to the original producer [172, 173]. Interestingly, the ration between epothilon A and B was 1:10 as opposed to a ratio of 2:1 in the original producer *Sorangium cellulosum* SMP44. Through a mutation of the epoK gene, epothilone C and D (the most cytotoxic derivate) could be produced as well.

Besides homologous recombination other techniques like Red/ET recombineering were employed to express the myxothiazol gene cluster with a concentration of 20 mg L^{-1} [168]. To overcome the disadvantage of a comparatively high metabolic background due to the production of endogeneous secondary metabolites, the gene cluster was cloned into the myxovirescin cluster of *M. xanthus*, thereby disrupting it and consequently reducing the metabolic burden. Myxothiazol A had been isolated from different strains of the genera *Angiococcus, Stigmatella* and *Myxococcus* and rarely from the genera *Cystobacter* and *Corallococcus*. Product titers in *M. xanthus* were comparable to that of the native producers *M. fulvus* Mx f16 (mg L^{-1}) and *Stigmatella aurantiaca* DW4-3/1 (10 mg L^{-1}) [167, 174]. Fu *et al.* transferred the myxochromide S gene cluster from *Stigmatella aurantiaca* DW4-3/1 into *M. xanthus* using transposition with a final titer of 500 mg L^{-1} which was 12.5 times higher than in *P. putida* [135, 166, 175]. The bengamides, originally from *Myxococcus virescens* ST200611 could also be produced in *M. xanthus* with a concentration comparable to the original producer (5-10 mg L^{-1}) [176, 177]. Aside from expression of pretubulysin in *P. putida*, the gene cluster was also integrated into *M. xanthus*. The exchange of the rare start codon TTG together with feeding of pipecolic acid could increase the titer of pretubulysin A to 190 μg L^{-1} which is more than tenfold the concentration achieved with *P. putida* [171]. Furthermore, various disorazol compounds were expressed

in *M. xanthus* with an overall yield of 400 μg L^{-1}. Disorazol A was the main derivative which is a five times higher concentration than in the original producer *Sorangium cellulosum* So ce12 [61, 178].

These studies show that *Myxococcus xanthus* DK1622 has proven a suitable host for expression of myxobacterial secondary metabolites with product titers comparable to or even better than those achieved by the original producers. The order of Myxococcales is very inhomogeneous containing strains with different nutrition and growth profiles. Production of their metabolites by large-scale fermentation is often necessary because of low titers but is hindered by the different demands posed by a variety of strains. These impediments could be overcome when using *Myxococcus xanthus* as an expression platform. But besides new techniques for gene transfer and engineering of the translation machinery, it is also necessary to develop and optimize cultivation of *M. xanthus* to maximize the product titer and promote its beneficial characteristics.

1.6 Systematic Production of Natural Products

1.6.1 Discovery and Production of Antibacterial Compounds from Myxobacteria

The discovery and production of antibacterial natural products from myxo- and other gliding bacteria follows a certain workflow that is depicted in Figure 1.15. First of all, the bacteria are isolated from soil samples. For this, the nutritional preferences of the different bacterial strains are harnessed. For the isolation of bacteriolytic myxobacteria, *E. coli* cells on water agar are used, while cellulose degraders are enriched on filter papers [30]. From there, single colonies or swarms can be isolated and be transferred in liquid culture. The culture is cultivated over several passages until a pure culture is obtained. This type of small scale cultivation (up to 100 mL) is usually done in shake flasks, but alternatives like 50 mL falcon tubes with aerated lids or deep-well micro-titer plates with covers like the Duetz-system are used as well [179]. The smaller cultivation volumes of these systems (around 10 mL) enable high throughput screening. On the other hand, gliding bacteria tend to stick to

the plastic walls of the smaller vessels which causes non-optimal nutrient and oxygen supply.

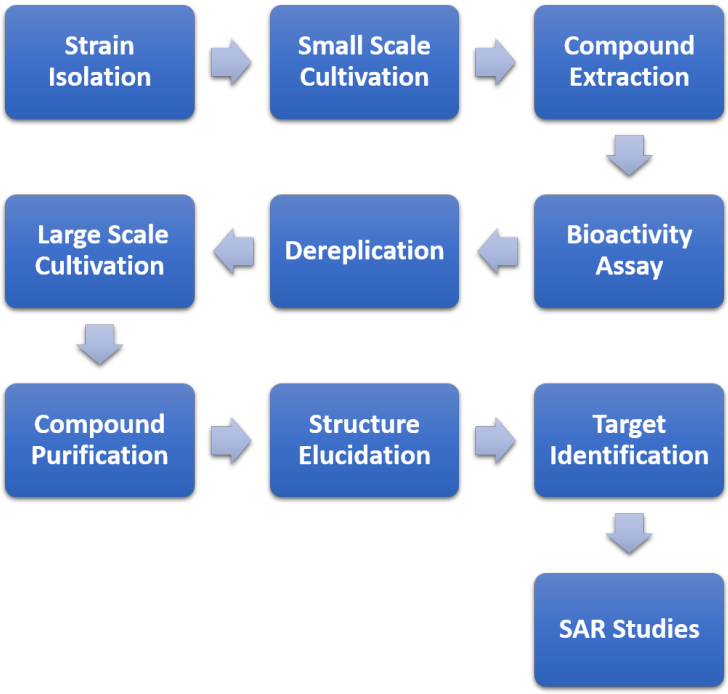

Figure 1.15: Typical workflow for discovery and production of antibacterial natural products originating from myxobacteria.

The compounds produced by the bacteria are invariably excreted to the culture medium and in most cases captured on the hydrophobic adsorber resin XAD16. The resin can be obtained through filtration and extracted with organic solvents, usually methanol or acetone. This crude extract contains all the compounds produced by the specific strain. It is subsequently subjected to a bioactivity assay where it is tested against several different organisms, including Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi. If the crude extract inhibits the growth of any of the test strains, it is fractionated with the help of a designated HPLC system and the fractions are tested against the respective organism once again.

In parallel, the crude extract is injected into a high-resolution HPLC-MS/MS system to record its mass spectrum. On the one hand, the retention time of the peak that inhibited the bacterial growth can be matched to its mass detected by the HPLC-MS/MS. On the other hand, the peaks' mass along with the retention time can be checked against a database of known compounds,

a process called dereplication. For myxobacteria, such a database was set up by the Müller group at the Helmholtz Centre for Pharmaceutical Research Saarland [180]. If the specific compound that caused the inhibition is yet unknown, the next step is a large scale cultivation in bioreactors to obtain a bigger amount for further testing. The subsequent experiments like structure elucidation, target identification and SAR studies require pure compounds, so that at this point the product needs to be purified from the crude extract by different chromatography methods. The pure compound is also included in a variety of test panels where it is tested for e.g. anti-viral or cytotoxic activities.

Despite being such a rich source of natural compounds, there are not many research groups around the world that study myxobacteria. This may origin from their undesirable features in terms of handling and cultivation. They have a very high doubling time with 4-14 h, compared to e.g. *E. coli* with 20 min [181]. Furthermore, they tend to form lumps and flakes when growing in liquid culture as well as sticking to only slightly rough surfaces, like stainless steel tubes or even bioreactor rotor blades. Additionally, most species do not feed on carbohydrates, but require complex peptide sources or starch as nutrients which calls for complex media and impedes mass balancing. However, one of the biggest challenges when dealing with myxobacteria are their low product titers which are usually in the low mg L^{-1} range. This leads to the fact that large-scale fermentations of 10-150 L cultivation volume are required for almost every product in order to obtain an adequate amount after purification for subsequent experiments.

1.6.2 Bioprocess Engineering

Bioreactor cultivations bear several advantages over shake flask cultivations. These are all founded in the fact that a bioreactor presents a controlled environment which increases the reproducibility of experiments drastically. For shake-flasks, the temperature can be controlled by setting a distinct temperature in the incubator. The usage of HEPES as a buffer is supposed

to keep the pH at a certain set-point. But in nearly every myxobacterial shake flask cultivation it can be observed that the pH becomes highly acidic or alkaline during the course of the cultivation. The main parameter that cannot be controlled in shake flasks is the oxygen supply. This is highly dependable on the shaking speed, the diameter of the shaking circle, the filling volume and the gas diffusion through the plug of the shake flask [182]. The first three parameters can be kept constant between experiments, the gas diffusion through the plug on the other hand differs between shake flasks. The main reasons are the type of plugs used and how well the plug fits on the shake flask [183].

Additionally, the oxygen transfer in shake flasks is generally lower than in bioreactors, because the gas transfer only occurs at the gas-liquid-interface [184]. The gas-liquid-interface in bioreactors is much higher because the air is supplied through a sparger that creates small bubbles (Figure 1.16). The gas flow can be also be controlled as well as the dissolved oxygen (DO) concentration which is measured by a designated DO probe. The DO can be kept at a certain set-point by regulation of the stirrer speed, gas flow rate or the addition of pure oxygen to the gas mix. The second probe common to all bioreactors is the pH probe which is connected to acid and base pumps through addition of which the pH can be kept at a constant level. One major feature of a bioreactor is the process control system with which time- or event-based profiles and online-monitoring for a variety of parameters can be realized. The most common profile is the feeding of nutrients after a certain cultivation time in a continuous or non-continuous manner. This type of cultivation process is called fed-batch fermentation.

Bioreactors exist in different sizes and a variety of configurations. One of the smallest commercially available single-use micro-scale bioreactor systems is the ambr® system of Sartorius Stedim with vessel sizes of 10-15 mL that comes with a liquid-handling system [186]. The parallel-cultivation system DASGIP® of the Eppendorf company represents a fully-equipped bench-top fermentation unit that allows exact pump rates due to microfluidic systems

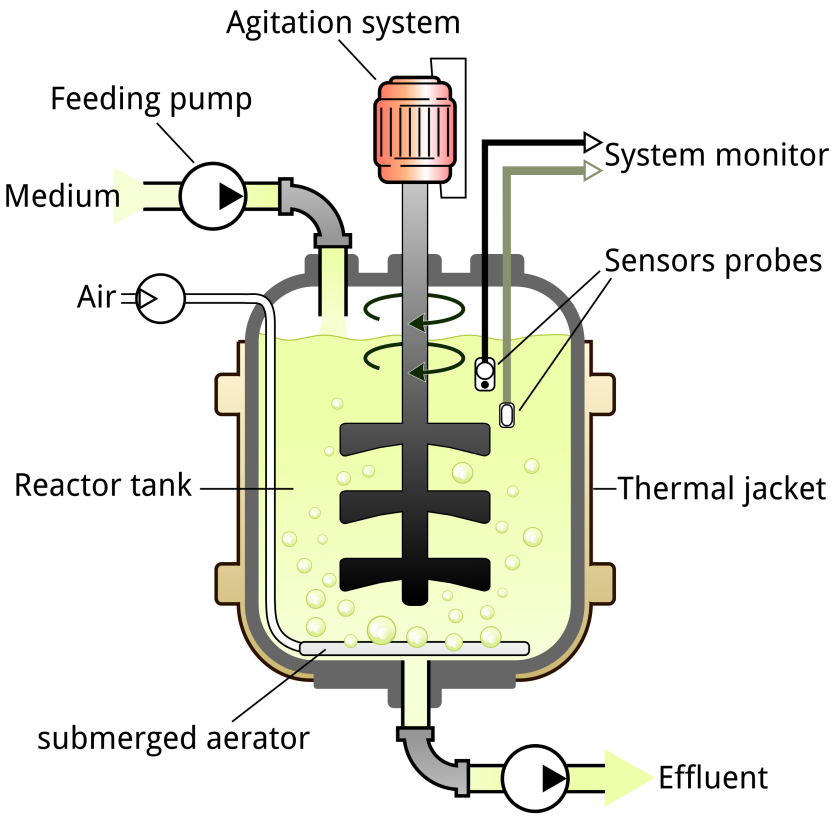

Figure 1.16: Schematic set-up of a bioreactor [185].

and parallel handling using an extensive process system (Figure 1.17). The smallest reactor volume available is 60 mL (for the compatible DASbox® system). *In situ* autoclavable stainless steel bioreactors are available from 10 L onwards up to several cubic meters. As the general build-up of bioreactors stays the same throughout all sizes, the scalability of bioprocesses mainly depends on the bioreactor geometry and the power input [187].

Despite the advantages of bioreactor systems, it can be assumed that probably more than 90 % of all culture experiments in biotechnology are performed in shaking vessels [188]. This is mostly based on the easy handling resulting in a high throughput. There are several companies tackling the problem of low amount of information retrieved from small-scale shaken systems. One solution is represented by the BioLector® system launched by m2p-labs which is basically an automated micro-titer plate cultivation system with online-monitoring of biomass, pH, DO, and fluorescences [189]. The Pro version is

Figure 1.17: Set-up of the parallel-cultivation system DASGIP®. The vessels are harbored in the so-called Bioblock that maintains the cultivation temperature. The gray blocks to either side are the modules responsible for parameter control. The process control system is operated via the DASware control software.

additionally equipped with continuous individual pH and feeding control. For online-monitoring of shake flask cultivations, the RAMOS (Respiratory Activity MOnitoring System) by the company HiTec Zang enables OTR (Oxygen Transfer Rate) and CTR (Carbon Dioxide Transfer Rate) measurements as well as feeding control for up to eight shake flasks in parallel [190]. These inventions enable a higher reproducibility of small-scale shaken systems with a higher information value and a more successful transfer of cultivation processes to large-scale stirred tank bioreactors.

As mentioned above, myxobacterial cultivations usually yield only a small amount of product. It is even more astonishing that only a few publications deal with engineering and optimization of myxobacterial bioprocesses. Nutritional requirements have already been studied as early as the 1960s but not under the aspect of secondary metabolite production [191, 192, 193]. Additionally, the production of myxobacterial products using large-scale fermentation has been reported for several products like corallopyronin, myxothiazol or the soraphens. But these processes were restricted to non-optimized batch fermentations [56, 194, 174]. The first account of optimization went along with the commercialization of epothilone production in the early 2000s which was implemented by heterologous expression in a *M. xanthus* strain. The product titer was improved 140-fold with the use of the adsorber resin

XAD16, the identification of a suitable carbon source and the implementation of a fed-batch process [173]. Through refinement of the fed-batch fermentation into an optimized semi-continuous culturing system with optimized bead retention times the yield could be increased even further [195]. In an effort to control the derivative distribution of epothilones, the importance of controlling the DO level at a certain set-point was revealed. At an excess oxygenation with a DO setpoint of 50 % epothilones A and B were the most abundant derivatives while an oxygen depletion lead to the accumulation of the pathway intermediates epothilones C and D [196], of which epothilone D was the desired product. This phenomenon can be attributed to the fact that epothilone D is processed by a cytochrome p450 monooxygenase to form epothilone B. As the activity of these enzymes is oxygen-dependent, it is hampered at low oxygen concentrations. The change of the metabolite profile at different DO levels could also be observed by Hüttel & Müller who optimized the fermentation of a *Chondromyces* strain using off-gas analysis [197].

Bioprocess engineering comprises the optimization of media components as well as fermentation parameters and feeding strategies. Optimization of the media composition is done in most cases in shake flasks by variation of one media component in each flask. This so-called "one-factor-at-a-time" approach bears the risk that the true optimal media composition is not reached because the interactions of the components are not taken into account (Fig. 1.18A) [198]. This problem is circumvented by using statistical experimental planning, also called "Design of Experiments" (DoE), the concept of mathematical relationships between input and output variables of a system (Fig. 1.18B). Therefore, DoE avoids experimental bias with a simultaneous reduction of experiments compared to the number that would be necessary when every factor would be combined with every other factor.

A full DoE optimization round contains three stages, as described in Figure 1.18C: In the first stage, important factors are identified through a set of experiments covering selected corners in the experimental space [200]. This

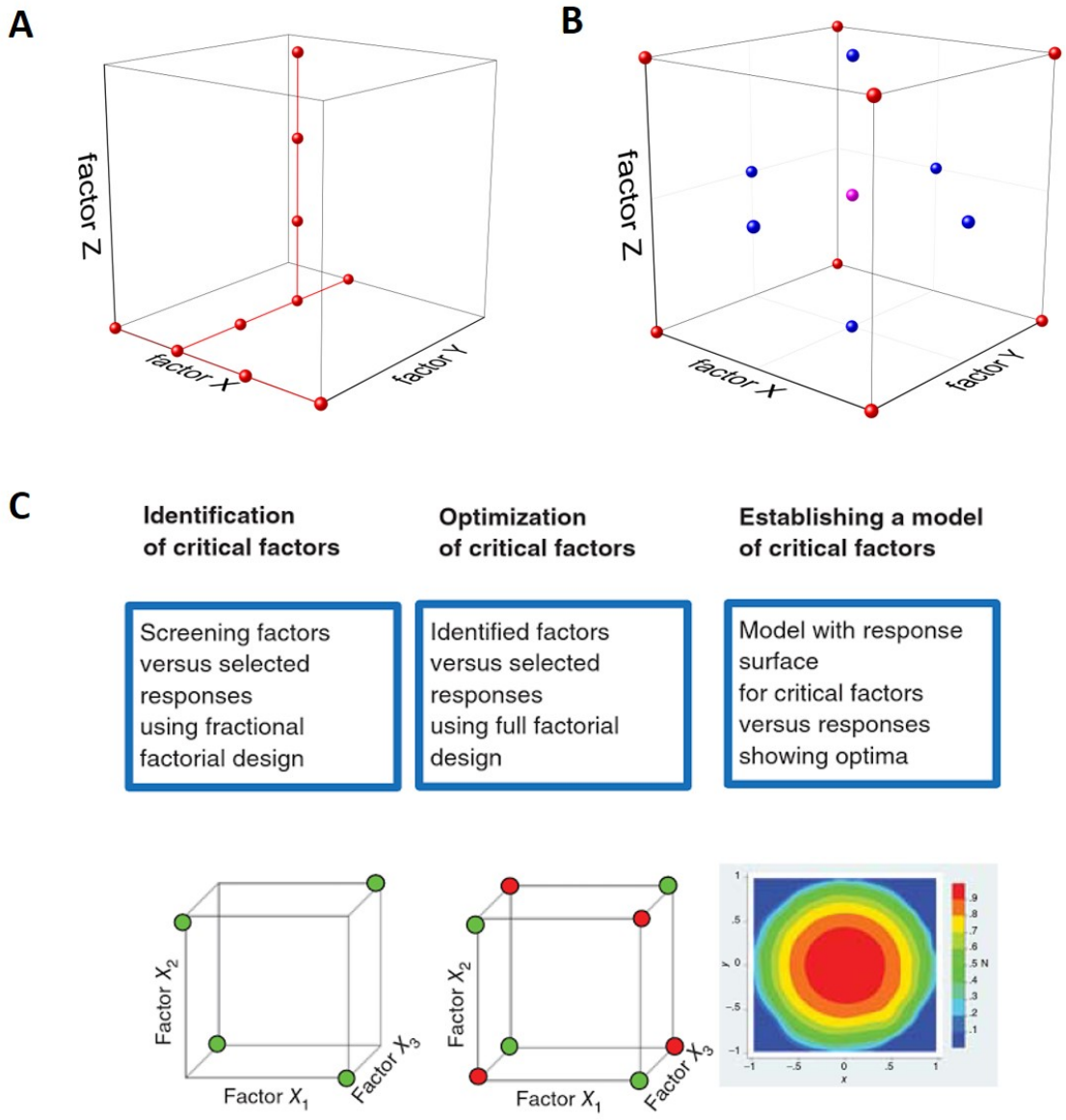

Figure 1.18: **A** The figure shows how a quasi-optimum is achieved by varying one variable at a time in an experimental setup with three factors. If only one factor is varied while the others are kept constant, a correct optimum would never be reached as there could be a dependency between the factors. **B** By simultaneous variations of the factors the whole experimental space can be covered, as shown on the basis of a response surface design. **C** The three stages of a full DoE round are shown including the designs recommended for every stage (adapted from [199]).

procedure is called fractional factorial design, since only a fraction of the possible values of the corners are investigated. This drastically lowers the number of experiments that is defined as $N = 2^{n-k}$, where n is the number of factors to be investigated and k is the number of steps to reduce the experimental design. In addition to the corner experiments, center point

experiments should be carried out in triplicate to determine the experimental error of the responses. Typically, a few important factors are identified in the first stage which are then optimized using a full factorial design that investigates all corners (2^n experiments). For further refinement and creation of a valid model a response surface methodology with additional experiments at the surface center points can be ensued as a third stage. The DoE approach can be carried out with every type of controlled factor, like media components or fermentation parameters. Additionally, the the influence of uncontrolled factors can also be taken into account.
Several research groups in the field of bioprocess engineering have reached promising results using the DoE approach. Wang and co-workers optimized the media composition for production of clavulanic acid with *Streptomyces clavuligerus* by first screening a variety of media ingredients by a two-level fractional factorial design approach which was followed by optimizing the levels of the media components by response surface methodology [201]. As a result, the product yield could be increased by 50 %. Roebuck *et al.* applied fractional factorial design and response surface modeling to determine the optimal temperature and pH for the growth of the yeast *Pachysolen tannophilus* [202]. An extensive overview of other applications is given in a review by Mandenius & Brundin [200].

1.6.3 Metabolic Engineering

Increase of product yield in bioprocesses can also be achieved by metabolic engineering techniques. These include targeted optimization of production rates such as controlling or enhancing expression of pathway genes or increasing the precursor supply for the biosynthesis of target compounds. Some of these approaches are presented in Figure 1.19.
One strategy focuses on the identification of regulators of secondary metabolite biosynthesis in the specific organism. Once this has been successful, targeted overexpression of positive or deactivation of negative regulators can follow resulting in an overproduction of the desired compound. However,

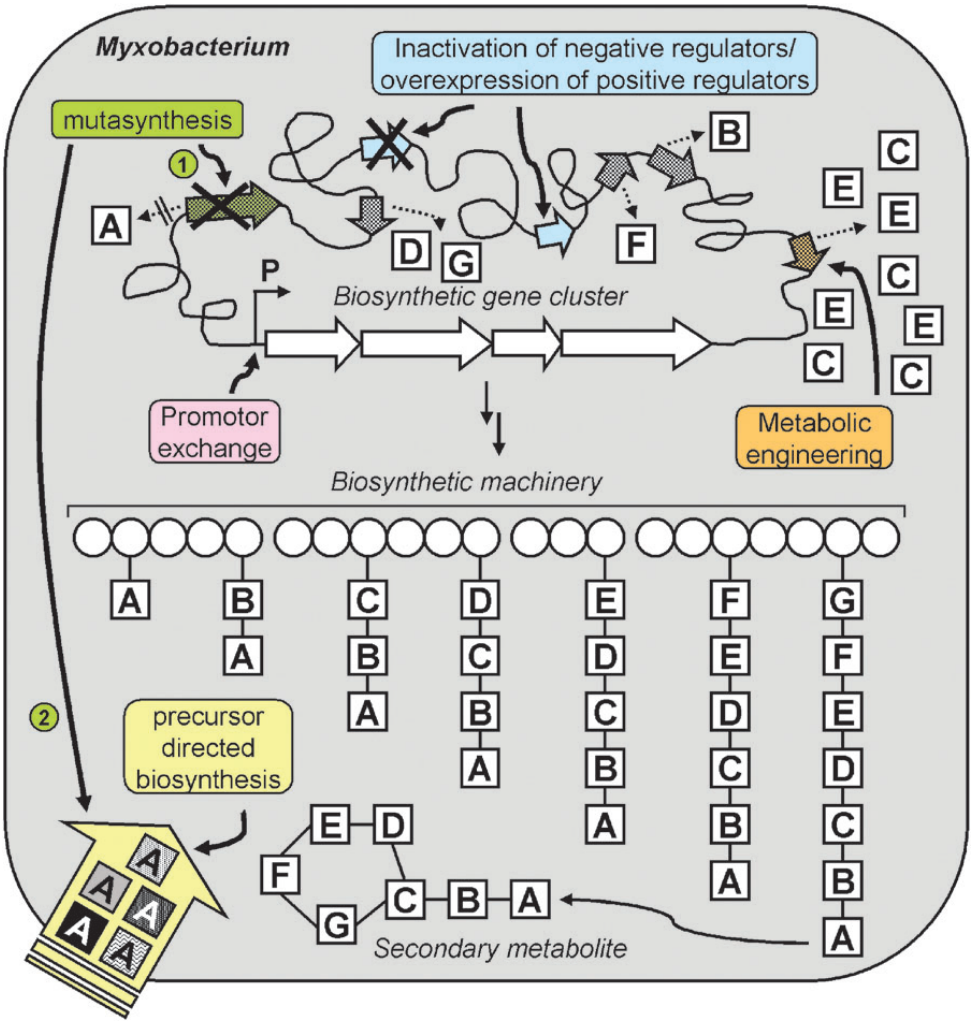

Figure 1.19: Schematic portrayal of genetic engineering approaches for secondary metabolite production in myxobacteria [203].

this approach is complicated by the fact that regulators of myxobacterial secondary metabolism are typically not co-localized with the biosynthetic genes, unlike the situation in actinobacteria [203]. Nevertheless, two regulatory proteins were discovered that bind specifically to the chivosazole gene cluster promoter region in *S. cellulosum* So ce56. ChiR was identified as a positive regulator, overexpression of which resulted in five-fold increased chivosazole titers [204]. NtcA on the other hand was shown to be a negative regulator. Its deactivation caused a four-fold increase in product yield [205]. The inactivation of negative regulators in *Angiococcus disciformis*, recently classified as *Archangium disciforme* [76], even led to a 30-fold higher concentration of the PKS/NRPS product myxothiazol [206]. An alterna-

tive strategy is the replacement of native by constitutive promoter elements. The introduction of the constitutive *tn5* promoter in front of a lipopeptide biosynthetic gene cluster in *M. xanthus* effectuated a 30-fold increase in the concentration of myxochromide A [203]. These examples show that targeted manipulation of the regulatory elements can be a powerful tool for bioprocess optimization. However, high product titers can only be achieved if there is no bottleneck in precursor supply. These include CoA-activated short chain carboxylic acids for PK and amino acids for NRP biosynthesis. This requires the analysis of metabolic fluxes as done with *S. cellulosum* So ce56 [207].
One major goal when producing and investigating natural products are SAR studies with the aim of understanding the effect of the functional groups of the molecule on pharmacokinetics and introducing new desirable properties. A simple approach of creating new compounds is the feeding of unnatural precursors to the fermentation culture which are taken up by the cells and are incorporated into the target molecules. This process is called "precursor-directed biosynthesis". Examples are fluorinated chondramide derivatives and various anti-bacterial elansolid derivatives [120, 208]. One drawback of this approach is that the unnatural precursors compete with the natural building blocks, leading to a compound mixture. This problem is circumvented by inactivation of the genes responsible for supply of starter units in combination with feeding of unnatural building blocks, termed as "mutasynthesis". By feeding various unnatural starter molecules to a *M. xanthus* mutant strain, it was possible to generate nine novel myxalamid derivatives [209]. Generation of new myxopyronin derivatives was also achieved by Sahner *et al.* for another *M. xanthus* mutant strain, although product titers were very low with 1-10 μg L^{-1} [210]. This studies lead to the ultimate aim of "combinatorial biosynthesis", the altercation of PKS/NRPS product molecular structures through editing of the biosynthesis gene cluster itself through genetic engineering. Although direct modifications of the PKS/NRPS backbone in myxobacteria has not been successful yet, promising results for other organisms exist [211]. However, inactivation of genes encoding post-assembly line process-

ing enzymes led to the formation of multiple derivatives in myxobacterial strains for the adjuzoles in *C. crocatus* [212], stigmatellin in *S. aurantiaca* [167] and spirangien in *S. cellulosum* [213]. As genetic engineering tools and techniques will expand and ameliorate in the future, these approaches could one day become the future of pharmaceutical research.

2 Material and Methods

2.1 Bacterial strains

For elansolid production, the strain *Chitinophaga sancti* Fx7914 was used.
For myxopyronin production, the myxopyronin A gene cluster was integrated into the genome of *Myxococcus xanthus* DK1622 ΔmchA-tet via Red/ET recombination according to [214]. The final strain used in this study was termed *M. xanthus* DK1622 ΔmchA-tet::pHSU-mxn43.
For vioprolide production, the vioprolide gene cluster was integrated into the genome of *Myxococcus xanthus* DK1622 (ΔmchA) via Red/ET recombination according to [215]. The final strain used in this study was termed *M. xanthus* DK1622 ΔmchA-tet::Ptn5-vio.

2.2 Media and supplements

Chitinophaga seed culture medium

For seed-cultures, medium (10.0 g L^{-1} soy peptone (Cargill), 1.0 g L^{-1} $CaCl_2 \cdot 2\ H_2O$, 1.0 g L^{-1} $MgSO_4 \cdot 7\ H_2O$, 8.0 mg L^{-1} Na-Fe-EDTA, 11.9 g L^{-1} HEPES; pH 7.4) was used to which maltose monohydrate was added after autoclaving to a final concentration of 10.5 g L^{-1}.

Chitinophaga production medium

Bioreactor cultivations were conducted with a production medium (1.99 g L^{-1} $(NH_4)_2SO_4$, 700 mg L^{-1} KH_2PO_4, 200 mg L^{-1} $MgSO_4 \cdot 7\ H_2O$, 8.0 mg L^{-1} Na-Fe-EDTA, 5.0 mg L^{-1} $MnSO_4 \cdot H_2O$, 1.0 mg L^{-1} $ZnCl_2$; pH 7.0) to which the carbon sources were added after autoclaving giving the following final concentrations: 10.0 g L^{-1} sucrose; 10.1 g L^{-1} glucose monohydrate. To enable elansolid C1 production an anthranilic acid solution in methanol was prepared through sterile filtration and added to the bioreactor before inoculation to give a final concentration of 100 μg L^{-1}. To capture the metabolites amberlite XAD16 resin (Sigma) was added to all production media to a concentration of 20 g L^{-1}. Feed solutions contained 440 g L^{-1} glucose monohydrate

and 146.7 g L^{-1} $(NH_4)_2SO_4$ that were autoclaved separately and afterwards merged to add up to 500 mL.

Myxococcus agar plate medium

For agar plates, a medium consisting of 10.0 g L^{-1} Bacto Casitone (Becton Dickinson), 1.97 g L^{-1} $MgSO_4 \cdot 7\ H_2O$, 136.1 mg L^{-1} KH_2PO_4, 1.21 g L^{-1} Tris, 15.0 g L^{-1} Bacto Agar (Becton Dickinson) (pH 7.4) was used. Sterile filtered kanamycin monosulfate was added to a final concentration of 50.0 mg L^{-1} after autoclaving.

Myxococcus seed culture medium

For seed cultures, a medium consisting of 10.0 g L^{-1} Bacto Casitone (Becton Dickinson), 1.97 g L^{-1} $MgSO_4 \cdot 7\ H_2O$, 136.1 mg L^{-1} KH_2PO_4, 11.9 g L^{-1} HEPES (pH 7.4) was used. Sterile filtered kanamycin monosulfate was added to a final concentration of 50.0 mg L^{-1} after autoclaving.

Myxopyronin production medium

The standard production medium consisted of 20 g L^{-1} skim milk powder (supplier unknown), 6.6 g L^{-1} glucose monohydrate, 4.0 g L^{-1} fructose, 2.0 g L^{-1} yeast extract (Ohly Kat), 1.0 g L^{-1} $CaCl_2 \cdot 2\ H_2O$, 1.0 g L^{-1} $MgSO_4 \cdot 7\ H_2O$ (pH 7.5). For testing different media components, the skim milk powder was substituted by 6.31 g L^{-1} lactose monohydrate and 14.0 g L^{-1} of either casein (Alfa Aesar), Bacto Casitone (Becton Dickinson), casamino acids (Becton Dickinson), bacteriological meat peptone (Organotechnie), yeast extract (Ohly Kat) or soy peptone (type II, Marcor). The bioreactor fermentations were either carried out with the standard production medium or with meat peptone production medium consisting of 20 g L^{-1} bacteriological meat peptone (Organotechnie) and 6.31 g L^{-1} lactose monohydrate instead of 20 g L^{-1} skim milk powder. For the fed-batch fermentation, the feed (500 mL) consisted of 60 g L^{-1} bacteriological meat peptone (Organotechnie), 6.31 g L^{-1} lactose monohydrate, 6.6 g L^{-1} glucose monohydrate, 4.0 g L^{-1} fructose, 2.0 g L^{-1} yeast extract (Ohly Kat), 1.0 g L^{-1} $CaCl_2 \cdot 2\ H_2O$, 1.0 g L^{-1} $MgSO_4 \cdot 7\ H_2O$ (pH

7.5). To every production medium sterile filtered components were added after autoclaving to final concentrations of 8.4 mg L^{-1} $FeCl_3 \cdot 6\ H_2O$, 0.1 mg L^{-1} vitamin B_{12} and 50.0 mg L^{-1} kanamycin monosulfate.

Vioprolide production medium

The standard production medium consisted of 20 g L^{-1} skim milk powder (supplier unknown), 6.6 g L^{-1} glucose monohydrate, 4.0 g L^{-1} fructose, 2.0 g L^{-1} yeast extract (Ohly Kat), 1.0 g L^{-1} $CaCl_2 \cdot 2\ H_2O$, 1.0 g L^{-1} $MgSO_4 \cdot 7\ H_2O$, 11.9 g L^{-1} HEPES (pH 7.5). For testing different media components, the skim milk powder was substituted by 6.31 g L^{-1} lactose monohydrate and 14.0 g L^{-1} of either casein (Alfa Aesar), Bacto Casitone (Becton Dickinson), bacteriological meat peptone (Organotechnie), yeast extract (Ohly Kat) or soy peptone (type II, Marcor). For bioreactor cultivations, no HEPES was used. To every production medium sterile filtered components were added after autoclaving to final concentrations of 8.4 mg L^{-1} $FeCl_3 \cdot 6\ H_2O$, 0.1 mg L^{-1} vitamin B12 and 50.0 mg L^{-1} kanamycin monosulfate, in addition to separately autoclaved amberlite XAD16 adsorber resin (Sigma) with a final concentration of 20 g L^{-1}. The supplemental amino acids were added to the production culture after 48 h with a final concentration of 100 mg L^{-1}.

2.3 Cultivation Conditions

Chitinophaga shake flask cultivations

250 mL shake flasks containing 100 mL pre-culture medium were inoculated with 1.8 mL cryo stock and incubated at 25 °C and 160 rpm for 24 h. From this seed-culture, bioreactors were inoculated to a concentration of 10 g L^{-1}.

Myxococcus shake flask cultivations

500 μL of a cryo stock were plated on an agar plate and incubated for 96 h at 30 °C. For seed culture, each 250 mL shake flask without baffles containing 50 mL seed-culture medium was inoculated with 5 agar plugs (each 0.5 mm in diameter). The seed-culture was incubated for 48 h at 160 rpm

and 30 °C. The production cultures (250 mL shake flask without baffles containing 50 mL production medium) were inoculated with 5 % seed-culture and were incubated for 148 h at 160 rpm and 30 °C.

Bioreactor cultivations

All bioreactor cultivations were performed with the parallel cultivation system DASGIP (Eppendorf) and DASware control 5 for process control. The vessels had a total volume of 2.0 L. The system was equipped with optical DO electrodes (Hamilton) and pH electrodes (Hamilton). The off-gas analyzer consisted of zirconium dioxide sensors for O_2 measurement and infrared sensors for CO_2 measurement (BlueSens). The DO concentration in the medium was kept at a constant level of 20 % by increasing the stirrer speed (200-1200 rpm) and adding pure oxygen to the gas mix if necessary, realized by an internal cascade. The gas flow rate was kept constant at 0.05 vvm. The pH level was kept constant as well by the addition of 50 g L^{-1} H_2SO_4 or 50 g L^{-1} acetic acid (for myxopyronin production) and 50 g L^{-1} KOH. When foaming occurred, Tegosipon anti foam (Evonik) was added with a 1 mL syringe.

For *Chitinophaga* cultivations, the pH was set at 7.0. The batch fermentations were started with a cultivation volume of 1.5 L and lasted 85 h. The fed-batch fermentations were started with a cultivation volume of 1.0 L and lasted 94-190 h. The feed was started after 48 h for the sucrose glucose fermentation and after 24 h for the glucose glucose fermentations with a feeding rate of $F=2.04 \cdot e^{0.056 \cdot t}$ mL h^{-1}.

For mxyopyronin and vioprolide production, the pH was set at 7.5. The cultivation volume was 1.5 L and the bioreactors were inoculated with 5 % seed-culture. In the myxoypronin fed-batch fermentation, the feed was started 96 h after inoculation and was kept at a constant speed of 5 mL h^{-1} for another 96 h.

2.4 Accompanying Analytics

Determination of optical density and substrate concentration

Optical density was determined with a Libra S11 photometer (Biochrom) at λ=600 nm. Samples were diluted to be in the linear range of the photometer. Sucrose concentrations and corresponding monomeric glucose and fructose were determined with a photometric assay kit from r-biopharm (art. no. 10716260035). For measuring of glucose and lactose concentration, a second method was applied using an Agilent 1260 series HPLC and a Phenomenex REZEX ROA-Organic Acid H^+ (8 %) column (300 mm x 7.8 mm x 8 μm) at 65 °C with a RID detector and an isocratic gradient of 0.05 mM H_2SO_4 for 45 min.

Determination of elansolid concentration

After sample taking the amberlite XAD16 was filtered through gauze. The residual resin was weighed and the 10-fold volume (w/v) of a 1 % acetic acid solution in acetone (v/v) was added to the resin. After 10 min of incubation on a horizontal shaker 1 mL supernatant was centrifuged at 20,000 g for 10 min. The supernatant was injected in to an Agilent 1200 Series HPLC with a Waters Acquity UPLC BEH C18 column (50 mm x 2.1 mm x 1.7 μm) at 40 °C using 0.01 % formic acid in water (v/v) and 0.01 % formic acid in acetonitrile (v/v) as mobile phase. The gradient went linearly from 5 % to 100 % acetonitrile in 20 min. The UV signals were detected at λ=220 nm. The product concentrations were determined via a calibration curve using 5 defined standard solutions.

Determination of myxopyronin concentration

1 mL of the culture was centrifuged at 20,000 g for 10 min. 500 μL of ice-cold methanol were added to 500 μL supernatant and the solution was mixed thoroughly. Afterwards, it was centrifuged again at 20,000 g for 5 min. The supernatant hereof was injected in to an Agilent 1200 Series HPLC with a Waters Acquity UPLC BEH C18 column (50 mm x 2.1 mm x 1.7 μm) at

40 °C using 0.01 % formic acid in water (v/v) and 0.01 % formic acid in acetonitrile (v/v) as mobile phase. The gradient went linearly from 5 % to 100 % acetonitrile in 20 min. The UV signals were detected at λ=220 nm. The product concentrations were determined via a calibration curve using five defined standard solutions.

Determination of vioprolide concentration

After sample taking the amberlite XAD16 was filtered through gauze. The residual resin was weighed and the 10-fold volume of methanol was added to the resin. After 30 min of incubation on a horizontal shaker 1 mL supernatant was centrifuged at 20,000 *g* for 10 min. The supernatant was injected in to an Agilent 1200 Series HPLC with a Waters Acquity UPLC BEH C18 column (50 mm x 2.1 mm x 1.7 μm) at 40 °C using 0.01 % formic acid in water (v/v) and 0.01 % formic acid in acetonitrile (v/v) as mobile phase. The gradient went linearly from 5 % to 100 % acetonitrile in 15 min. The UV signals were detected at λ=220 nm. The product concentrations were determined via a calibration curve using 5 defined standard solutions.

Serial dilution assay for determination of antibacterial activity

Minimum inhibitory concentration (MIC) values were determined in standard microbroth dilution assays as described in [216]. In brief, bacterial suspensions of *Escherichia coli* DSM-1116 and TolC-deficient *E. coli* were prepared in Müller-Hinton broth (104-105 cfu mL^{-1}). In addition, the efflux-deficient *E. coli* strain was permeabilized with polymyxin B nonapeptide (PMBN) at a sub-inhibitory concentration (3 μg mL^{-1}) and used for testing following the same procedure. Given MIC values are the lowest concentrations of antibiotic at which there was no visible growth.

2.5 Design of Experiments Setup

For the setup and evaluation of the Design of Experiments runs, the software MODDE (Version 9.0.0.0) was used. Since the goal of these experiments

was the screening of different fermentation parameters, a fractional factorial design was used. Using this design with four factors resulted in eight runs plus three center point-runs (Table 3.2). The center points are the mean values of the extreme values of the investigated factors.

3 Results

The aim of this thesis is the investigation of the production capacity of myxobacteria and gliding bacteria of the genus *Chitinophaga*. As stated above, there is a need for novel drugs for the treatment of infectious and other diseases that are only partially met by compounds of synthetical origin. Thus, natural compounds with desirable properties and promising modes-of-action are indispensable if a "post-antibiotic era" should be avoided. Myxo- and other gliding bacteria play an important role as producers of bioactive compounds with new modes-of-action. The demand for sophisticated handling of these bacteria due to long cultivation times and special nutritional requirements calls for a certain expertise that leads to the fact that in the past myxobacteria have been neglected by the pharmaceutical industry as sources of new drugs. One point that plays a major role in this context are low product titers achieved through bioreactor cultivation of these organisms. In this thesis, the problem of low product titers is tackled in three different projects.

The first project covers the biotechnological production of the compound group of elansolids, anti-MRSA agents produced by the gliding soil-dwelling bacterium *Chitinophaga sancti*. The other two projects deal with the heterologous production of PKS- and NRPS-derived molecules with the model myxobacterium *Myxococcus xanthus*. The investigation of possible advantages of heterologous expression over production with the natural producer is thought to be a step towards the use of *M. xanthus* as an universal expression platform for certain classes of natural compounds.

With the exception of the elansolid bioactivity assay which was carried out by Viktoria Schmitt at the Helmholtz Institute of Pharmaceutical Research Saarland, all of the experiments were planned, performed and evaluated by the author of this thesis.

3.1 Production of Elansolids with *Chitinophaga sancti* Fx7914

To turn the highly interesting class of elansolids (see Chapter 1.3.1) into a lead for pharmaceutical development, it will be necessary to improve both their stability and potency. This compound class is amenable to various structural modifications by means of medicinal chemistry. However, for this purpose, the yields from biotechnological production need to be improved, in order to provide access to the starting material in multi gram scale. Elansolid A3, which has hitherto been used as starting material for semi-synthesis approaches is highly unstable. The rationale to direct the biotechnological production in order to allow for easy access of the starting material for a subsequent medicinal chemistry program is that it should be more feasible to produce elansolid A2. The latter compound can be converted back to elansolid A3 under mild conditions [105, 119]. For this reason, the biotechnological production of elansolid A2 with *C. sancti* was investigated in this study. One drawback of the biotechnological production has so far been the formation of several side products – especially in complex media - due to the reactivity of the elansolids A1-A3, which significantly lowered the yield of elansolid A2 in such a process. The reduced yield in undefined media arises from unspecific side reactions with either nucleophilic media components or intermediates, which are released by the producer itself due to cell lysis occuring in complex media when toxic metabolites accumulate. This might be circumvented by reducing formation of toxic metabolites and increasing the viability of the producer with the usage of a defined media and by concurrently directing the product formation towards a stable derivative through feeding of the corresponding precursor. The feasibility of this precursor-directed approach was explored in this study by feeding anthranilic acid, the precursor of elansolid C1 (6) which was shown to be incorporated *in vivo* and could thereby serve as a model system for direct incorporation of selected building blocks during the production process.

In previous studies, the activity spectrum of the elansolids was determined to include only Gram-positive bacteria, like *Staphylococcus aureus*. To val-

idate if the molecular target of the elansolids also exists in Gram-negative organisms and activity is reduced by reduced membrane diffusion or efflux, a bioactivity assay with the secretion-deficient *E. coli* tolC in combination with the membrane-permeabilizing peptide PMBN was performed revealing that elansolids A2 and C1 inhibit this gram-negative strain with 2 μg mL^{-1} and 8 μg mL^{-1}, respectively (Table 3.1). Following this logic, the elansolids presumably act on the same molecular target in gram-positive and gram-negative bacteria, but cannot penetrate the membrane of Gram-negatives [217].

Table 3.1: Minimum inhibitory concentrations (MIC) in μg mL^{-1} of elansolids A2 and C1 against different *E. coli* strains.

Strain	**Elansolid A2**	**Elansolid C1**
E. coli DSM1116	>64	>64
E. coli TolC	64	64
E. coli TolC+PMBN	2	8

According to Steinmetz *et al.*, it is crucial to use a defined medium without complex media components when producing elansolid A2 because otherwise elansolid C1, a derivative containing anthranilic acid, is produced instead of elansolid A2 [120]. A defined medium was developed for which sucrose was found to be the most suitable carbon source for elansolid A2 production during shake flask experiments with an elansolid A2 concentration of around 18 mg L^{-1} (data not shown).

The results were transferred to bioreactor scale, where a batch fermentation in the minimal medium with sucrose as carbon source was conducted (Figure 3.1). The disaccharide sucrose was metabolized right from the beginning, but released fructose accumulated in the medium until 61 h after inoculation when the concentration had its highest value with 2.7 g L^{-1}. Released glucose could only be detected at the beginning of the fermentation with a concentration of 0.18 g L^{-1} until 13 h (0.03 g L^{-1}) after inoculation but vanished within less than 24 h. This indicates that sucrose was probably digested by extracellular invertases and glucose was taken up preferentially by the cells until sucrose was depleted. Afterwards, the remaining fructose

was metabolized. After 18 h, a change from exponential to limited growth could be observed, as indicated by the CTR (carbon dioxide transfer rate) (Figure 3.1a). This might be due to cleavage of sucrose and supply of glucose as a limiting factor. After 37 h elansolid A2 production could be detected with a sigmoidal increase until the end of the cultivation to a final value of 18.9 mg L^{-1} comparable to the shake flask experiments. As illustrated in Figure 3.1c, elansolid A2 is not the only derivative produced. In fact, it accounted for only 14 % of the total elansolid yield based on the peak areas of the HPLC runs where the sum of all peak areas is accounted for as 100 %. The main product is elansolid B1 (water addition to elansolid A3). Although elansolid A3 is unstable in an aqueous environment it can still be detected, presumably due to slow reaction kinetics or possible protection when bound to the adsorber resin. Besides the published elansolid derivatives there are also several others, which are detectable in minor amounts but together make up a substantial fraction of the overall elansolid yield.

The batch process with sucrose did not lead to an increase in elansolid A2 concentration compared to the shake flask cultivation. Hence, as a next step towards the aim of improved elansolid A2 yield, a fed-batch fermentation was conducted. Sucrose was provided in the medium at the beginning of the fermentation with a concentration of 20 g L^{-1}, using the starting conditions of the batch fermentation. Due to preferred utilization of glucose by *C. sancti* in the previous experiment, this sugar was chosen as substrate in the feeding solution. The fermentation was started as a batch cultivation and after 48 h an exponentially increasing glucose feed was started. As seen in Figure 3.2, the curve-progression of the illustrated parameters develops similarly to the batch fermentation until the feed was started after 48 h, also exhibiting the transition from exponential to linear growth after 18 h. After starting the feed the CTR rapidly increased from 2.4 mMol h^{-1} to 12.3 mMol h^{-1} after 59 h. The elansolid A2 concentration increased until 74 h to 55.3 mg L^{-1} resulting in a 2.9-fold increase in comparison to the batch fermentation. As in the batch fermentation, several elansolid derivatives were produced of which elansolid

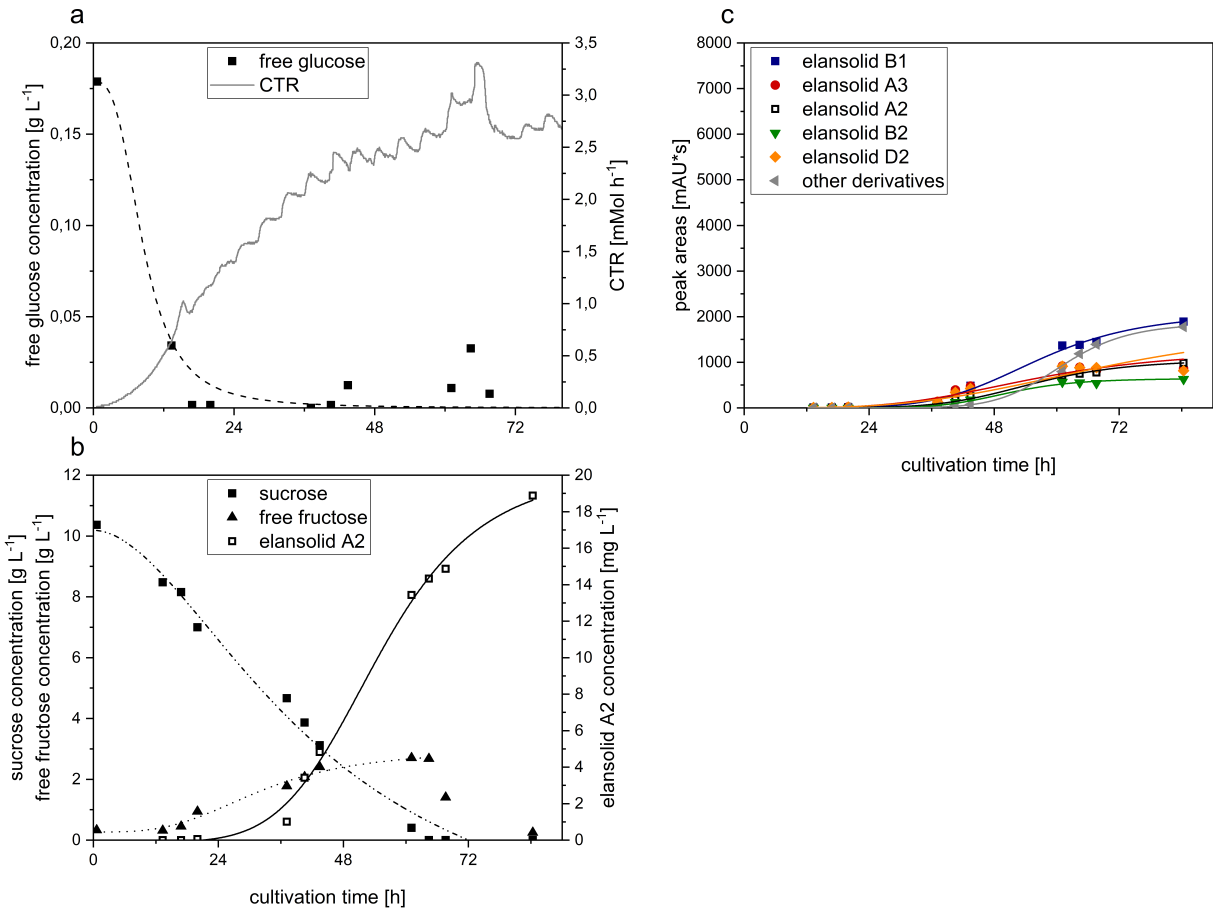

Figure 3.1: **a** Course of carbon dioxide transfer rate (CTR), free glucose concentration and **b** sucrose, free fructose and elansolid A2 concentrations and **c** peak areas of elansolid derivatives from the HPLC runs throughout the bioreactor batch fermentation of *C. sancti* with sucrose as carbon source.

A2 made up only 15 % of the overall yield (Figure 3.2c).

Since sucrose as carbon source caused a diauxic growth of *C. sancti*, a second fed-batch fermentation with glucose as sole carbon source in batch and feed-phase was conducted (Figure 3.3). The feed was started after 24 h. The CTR increased exponentially in the first 28 h after which a small dent occurred which correlates with the starting point of elansolid A2 production. Following the dent, the CTR increased linearly to 21.0 mMol h^{-1} in average from 48 h to 116 h. The oscillation of the CTR is caused by the fact that whenever the culture becomes more acidic, carbon dioxide that was formerly dissolved in the medium is released. When the pH is regulated by adding potassium hydroxide more carbon dioxide can be dissolved again. Therefore, these

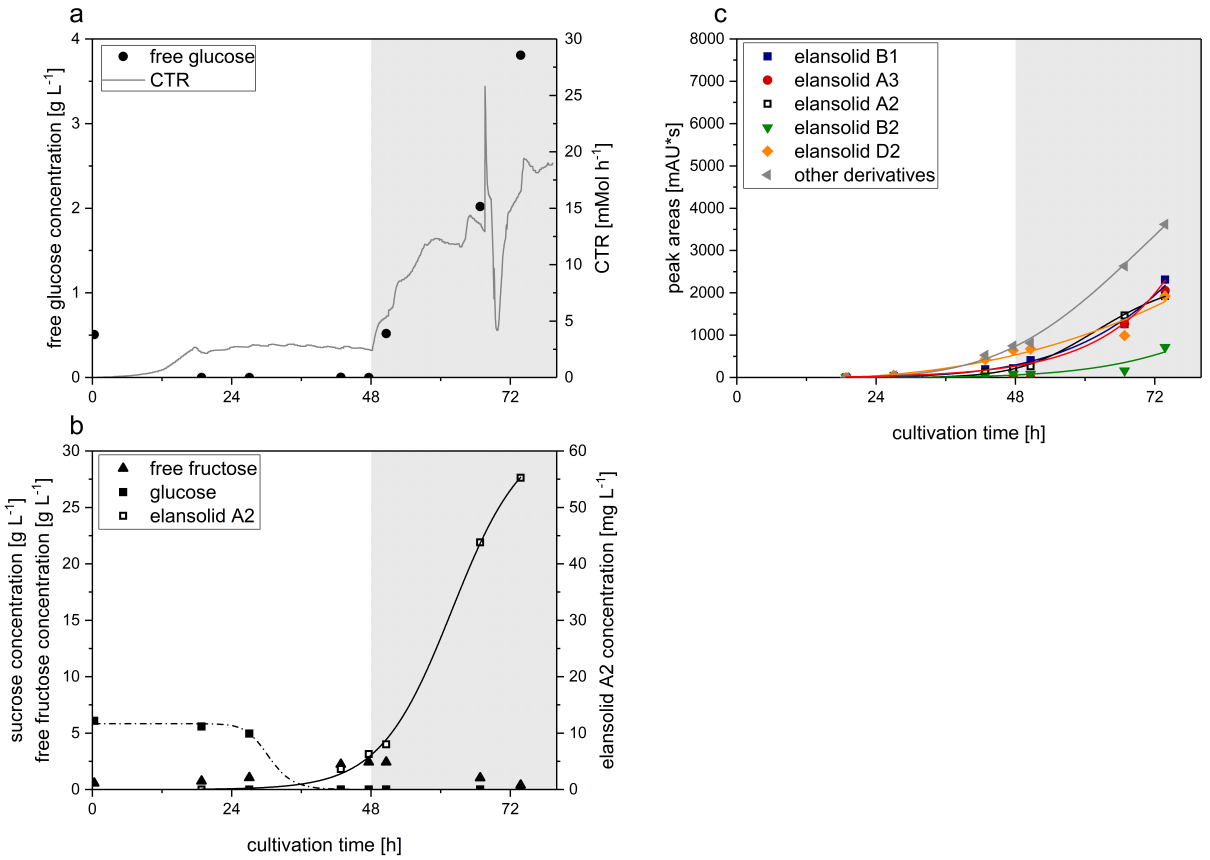

Figure 3.2: **a** Course of carbon dioxide transfer rate (CTR) and free glucose concentration, **b** sucrose, free fructose and elansolid A2 concentrations, **c** peak areas of elansolid derivatives from the HPLC runs throughout the bioreactor fed-batch fermentation with sucrose in the basal medium and glucose in the feed solution. The gray areas represent the duration of feeding.

fluctuations do not represent the true carbon dioxide emission rate (CER), a problem that has been addressed by several publications before [218, 219, 220]. The elansolid A2 concentration increased to 34.4 mg L^{-1} after 67 h which was less than in the fermentation with sucrose in the starting medium (end concentration was 31.8 mg L^{-1}). This is due to the fact that elansolid C1 was detected in high concentrations from 67 h onwards which was so far only encountered when complex substrates were present in the medium [120]. The final concentration of elansolid C1 was 223.1 mg L^{-1} and accounted for 32 % of all derivatives.

The accumulation of undesired side products in all cultivations for elansolid A2 production justified the investigation of a process aiming for the exclusive

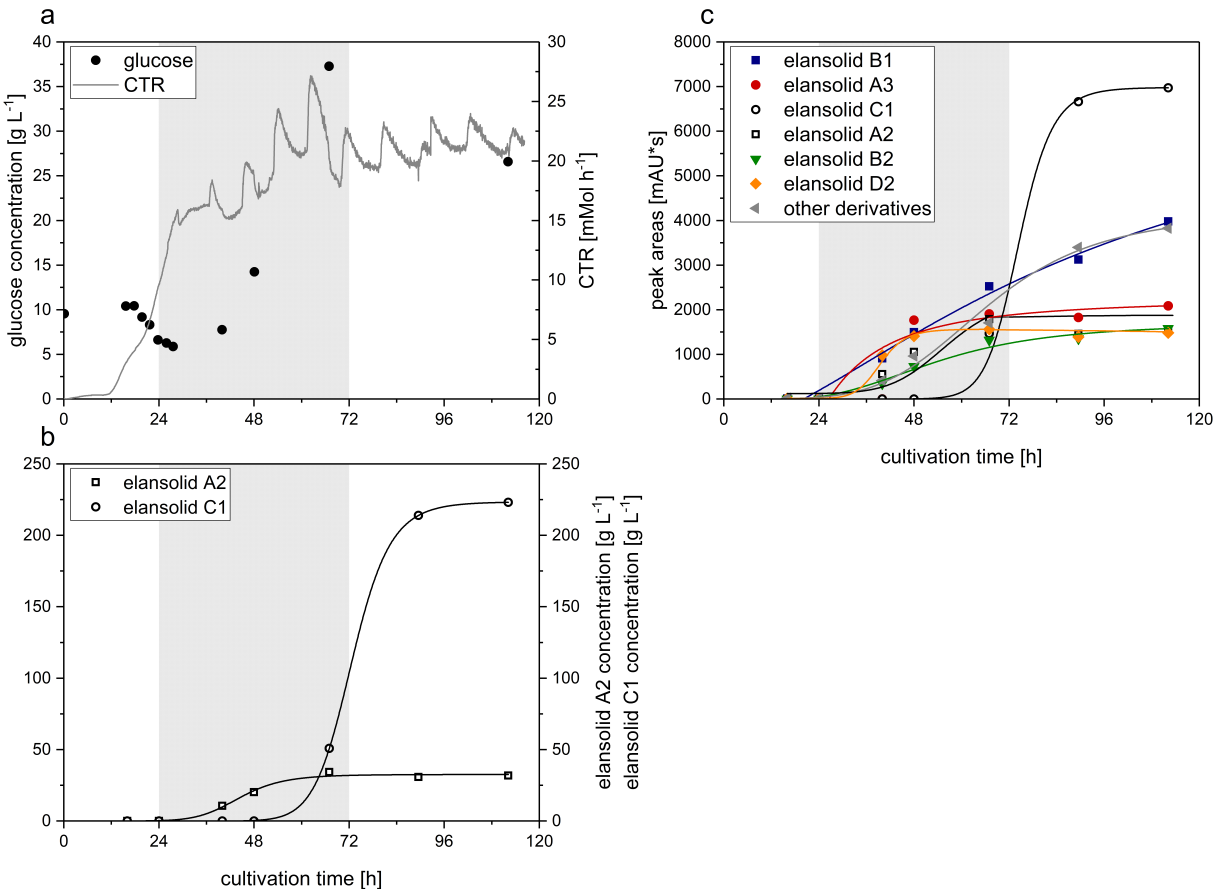

Figure 3.3: **a** Course of carbon dioxide transfer rate (CTR) and free glucose concentration, **b** sucrose, free fructose and elansolid A2 concentrations, **c** peak areas of elansolid derivatives from the HPLC runs throughout the bioreactor fed-batch fermentation with glucose in the basal medium and glucose in the feed solution. The gray areas represent the duration of feeding.

production of a single derivative. As a model system elansolid C1 was chosen since the precursor is readily available and C1 has similar activities as A1/A2 [120]. So far, the directed biosynthesis of elansolid C1 with complex medium yielded product titers with concentrations of around 7.1 mg L^{-1} in batch-fermentations [120]. To show the feasibility of the directed biosynthesis of elansolid C1 in defined medium, a fed-batch fermentation with anthranilic acid supplementation to the medium was conducted. The results are shown in Figure 3.4. The respirational activity, represented by the CTR, reached an average of 14.3 mMol h^{-1} from 48 to 120 and therefore only 68 % of the CTR of the fed-batch with glucose as sole carbon source without anthranilic acid (Figure 3.3). As a consequence of the reduced metabolic activity, glucose

accumulated to a final concentration of 77.5 mg L^{-1}. First elansolid C1 was detected after 17 h followed by an exponential increase with a maximum titer of 252.7 mg L^{-1} after 115 h. In this fermentation, the desired derivative elansolid C1 made up the major portion of the total yield with 85 % based on the peak areas.

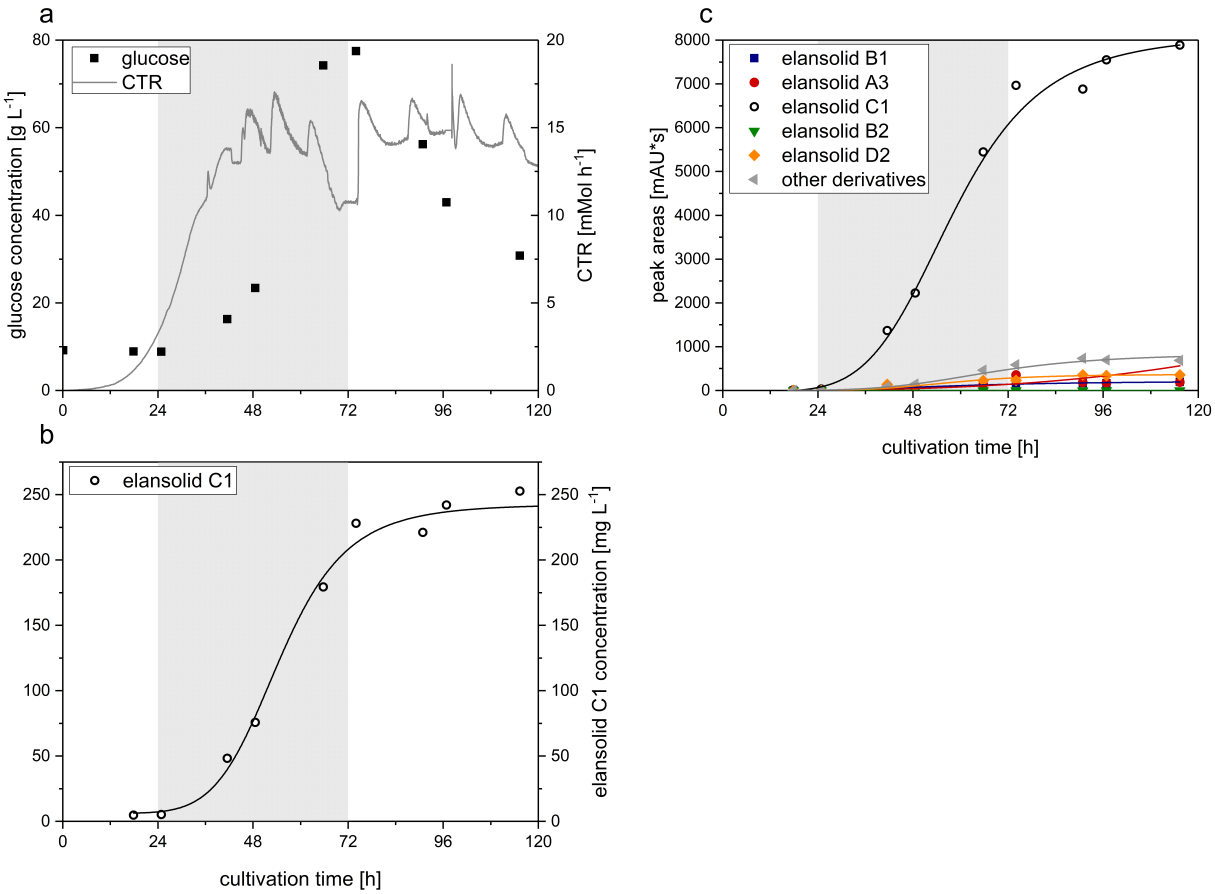

Figure 3.4: **a** Course of carbon dioxide transfer rate (CTR) and free glucose concentration, **b** sucrose, free fructose and elansolid A2 concentrations, **c** peak areas of elansolid derivatives from the HPLC runs throughout the bioreactor fed-batch fermentation with glucose and anthranilic acid in the basal medium and glucose in the feed solution. The gray areas represent the duration of feeding.

3.2 Heterologous Production of Myxobacterial Products with *Myxococcus xanthus* DK1622

One major drawback of myxobacterial compounds on their way to being developed as marketable products are the low product titers obtained from the

original producers. In an attempt to increase the production, the gene clusters of several myxobacterial products were cloned into different host organisms, e.g. *Streptomyces lividans* [159], *Escherichia coli* [163], *Pseudomonas putida* [168, 171] and *Myxococcus xanthus* [175, 177, 178]. *M. xanthus* is the most-studied myxobacterium and combines several advantages for the heterologous expression of myxobacterial natural products through the close genetical relatedness. The most important requirements for functional expression of these compounds are considered to be a similar codon usage, recognition of all gene cluster elements, precursor supply and provision of enzymes for the activation of the biosynthetic machinery [155, 156, 157]. After successes with other myxobacterial products where product titers were throughout higher than in the natural producers [175, 177, 178], the myxopyronin and the vioprolide biosynthetic gene clusters were cloned into *M. xanthus* DK1622 [214, 215].

3.2.1 Heterologous Production of Myxopyronin A

The order Myxococcales is very diverse and comprises genera with a variety of nutritional requirements. Species of the genus *Myxococcus* can only metabolize peptides as they feed on other bacterial cells in their natural environment [192, 193]. Thus, when using *Myxococcus xanthus* as an expression host, one has to create a medium that combines optimal cell growth with desirable physicochemical characteristics. The medium that was used for the initial shake flaks cultivations of *M. xanthus* DK1622 contains skim milk which consequently leads to a turbid medium that also coagulated during autoclaving and therefore is not suitable for large-scale cultivation. Thus, one focus of this study was the development of a nutrition medium without any non-soluble components to facilitate bioreactor cultivation and compound purification.

Besides skim milk powder, which was the original CN-source, various components of skim milk in different hydrolyzation grades were tested, as well as meat peptone and peptones derived from non-animal origin. Through HPLC

measurement, it could be shown that the skim milk powder consisted of 30 % lactose (data not shown). In order to keep the composition of the different media as equal as possible, the 20 g L^{-1} skim milk were substituted with 6 g L^{-1} lactose and 14 g L^{-1} of the complex CN-source. For the evaluation of the effect of different CN-sources on the product concentrations, shake flask cultivations were performed, of which the results can be seen in Figure 3.5.

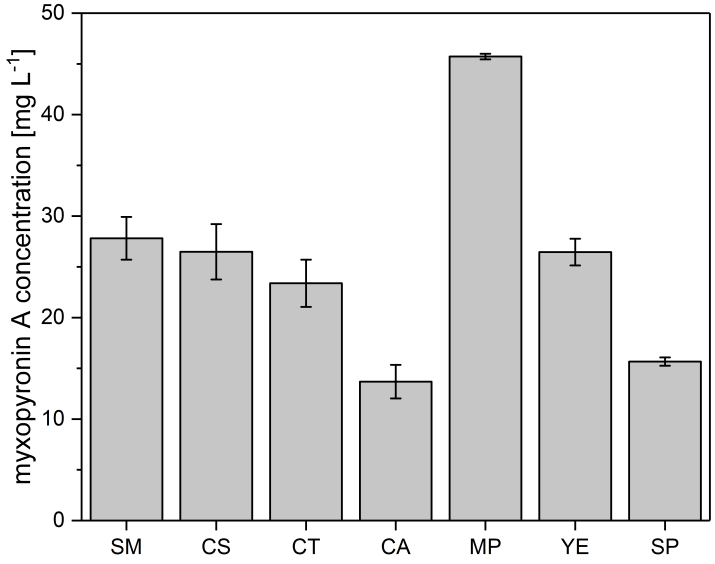

Figure 3.5: Myxopyronin A concentration of shake flaks cultivations after a cultivation time of 148 h for media with different complex CN-sources. The cultivations were done in triplicates; the error bars represent the standard deviation. SM=skim milk, CS=casein, CT=casitone, CA=casamino acids, MP=meat peptone, YE=yeast extract, SP=soy peptone.

The use of the standard medium containing skim milk (SM) resulted in a myxopyronin A concentration of 27.8 mg L^{-1}, similar to this when using casein (CS) (26.5 mg L^{-1}). Casitone is the pancreatic digest of casein and its use yielded 23.4 mg L^{-1}, whereas the provision of casamino acids, which is obtained by acidic hydrolysis of casein yielded only 13.7 mg L^{-1}. In contrast, the highest myxopyronin A concentration was obtained by the use of meat peptone, with a final concentration of 45.7 mg L^{-1}. The two non-animal peptones yeast extract (a yeast autolysate) and soy peptone (enzymatic digest of soy flour) yielded final product concentrations of 24.5 mg L^{-1} and 15.7 mg L^{-1}, respectively.

In parallel, a Design of Experiment (DoE) study was conducted aiming at

the screening of cultivation parameters with regards to their influence on the product concentration. The investigated parameters were cultivation temperature, pH, DO (dissolved oxygen) concentration and air flow rate. As experimental setup, a fractional factorial design was chosen which resulted in eight fermentation runs with different parameter combinations plus three center point runs. The parameter settings for each fermentation are shown in Table 3.2. The temperature range was chosen to be from 25-35 °C, the pH range from 6.5 to 8.5, the DO setpoint range from 5-40 % and the range of the air flow rate from 0.01 to 0.1 vvm. The center points, represented by the runs N9-N11, are the mean values of the extreme values of each investigated parameter. In Figure 3.6, the maximum myxopyronin A concentrations for the 11 DoE fermentation runs are presented.

Table 3.2: Parameter settings for the 11 DoE fermentation runs for the production of myxopyronin A.

Run No.	**Temperature** [°C]	**pH setpoint** [-]	**DO setpoint** [%]	**Air flow rate** [vvm]
N1	25	6.5	5	0.01
N2	35	6.5	5	0.1
N3	25	8.5	5	0.1
N4	35	8.5	5	0.01
N5	25	6.5	40	0.1
N6	35	6.5	40	0.01
N7	25	8.5	40	0.01
N8	35	8.5	40	0.1
N9	30	7.5	22.5	0.055
N10	30	7.5	22.5	0.055
N11	30	7.5	22.5	0.055

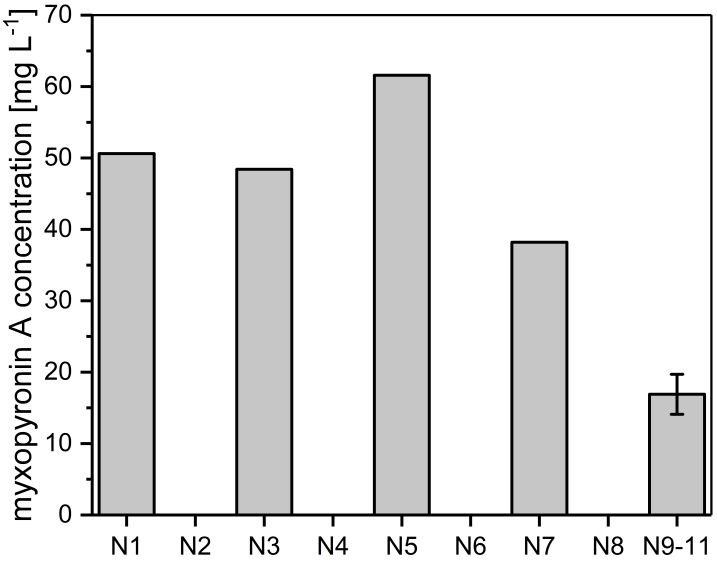

Figure 3.6: Maximum myxopyronin A concentrations of the DoE fermentation runs. The runs N2, N4, N6 and N8 did not yield any product. The error bar represents the standard deviation of the three center point runs N9-N11.

The center point runs N9-N11 are clearly in the same product concentration range with a mean value of 16.9 ± 2.8 mg L^{-1} myxopyronin A after 91.3 h at which time point the highest product concentrations of all three runs could be measured. During the further course of the fermentation, the concentrations decreased, probably due to instability of the product. The runs at 25 °C cultivation temperature yielded between 37.4 mg L^{-1} (N7) and 61.6 mg L^{-1} (N5) myxopyronin A. The product formation kinetics of runs N3 and N7 were comparable to those of the center point runs, whereas those of runs N1 and N5 were much slower with maximum product concentrations after 210.5 h and 243.6 h, respectively. This fact lets one assume that the process parameters other than the temperature have an influence on the product formation or maximum concentration. However, when looking at the DoE model that was created by the software, it can be seen that the temperature is the only factor that has a significant influence on the product concentration (Figure 3.7). The influence of the other factors is smaller than the standard deviation calculated from the center point runs and is therefore not significant. With temperature as the only significant factor, the model created has a coefficient of determination of R^2=0.91.

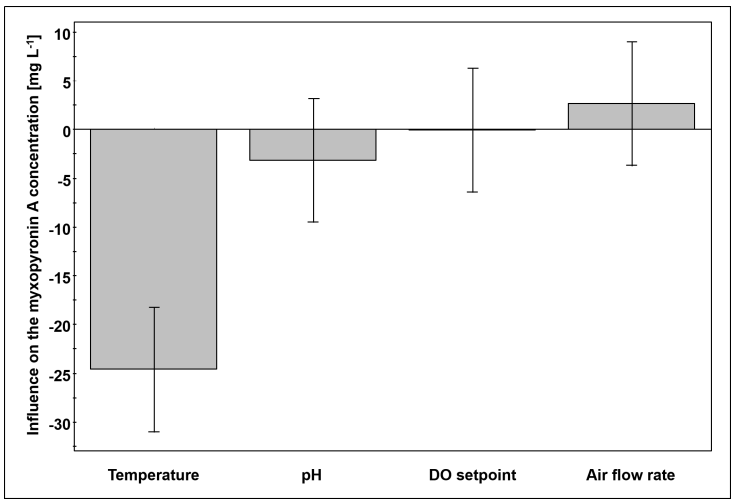

Figure 3.7: Influence of the different cultivation parameters on the myxopyronin A concentration according to the DoE model. The error bars represent the standard deviation of the three center point runs.

As the DoE showed a positive influence of lower temperatures on the myxopyronin A concentration, fermentations with the standard medium and the standard parameters at different temperatures were conducted. The results are shown in Figure 3.8.

The outcome of this experiment strengthens the results of the DoE runs. The highest product concentration (86.9 mg L^{-1}) could be achieved at a cultivation temperature of 20 °C, followed by a maximum concentration of 68.1 mg L^{-1} at 22 °C, 54.0 mg L^{-1} at 25 °C and 36.6 mg L^{-1} at 28 °C. For online monitoring of the growth of a culture, the carbon dioxide transfer rate (CTR) can be used. Unfortunately, the CTR in these parallel cultivations was only recorded for 120 h due to a technical defect. For further observation of the cultures' growth over the whole cultivation time, the amount of added sulfuric acid was recorded (Figure 3.8C). This can also be correlated to the growth because the catabolism of amino acid results in the accumulation of ammonia which is exported by the bacteria. In order to keep the pH at the given set point, sulfuric acid is added. Thus, the more peptides are catabolized, the more ammonia is built and the more sulfuric acid is added to the culture. When comparing growth kinetics with product formation kinetics, a correlation between growth and product formation seems likely.

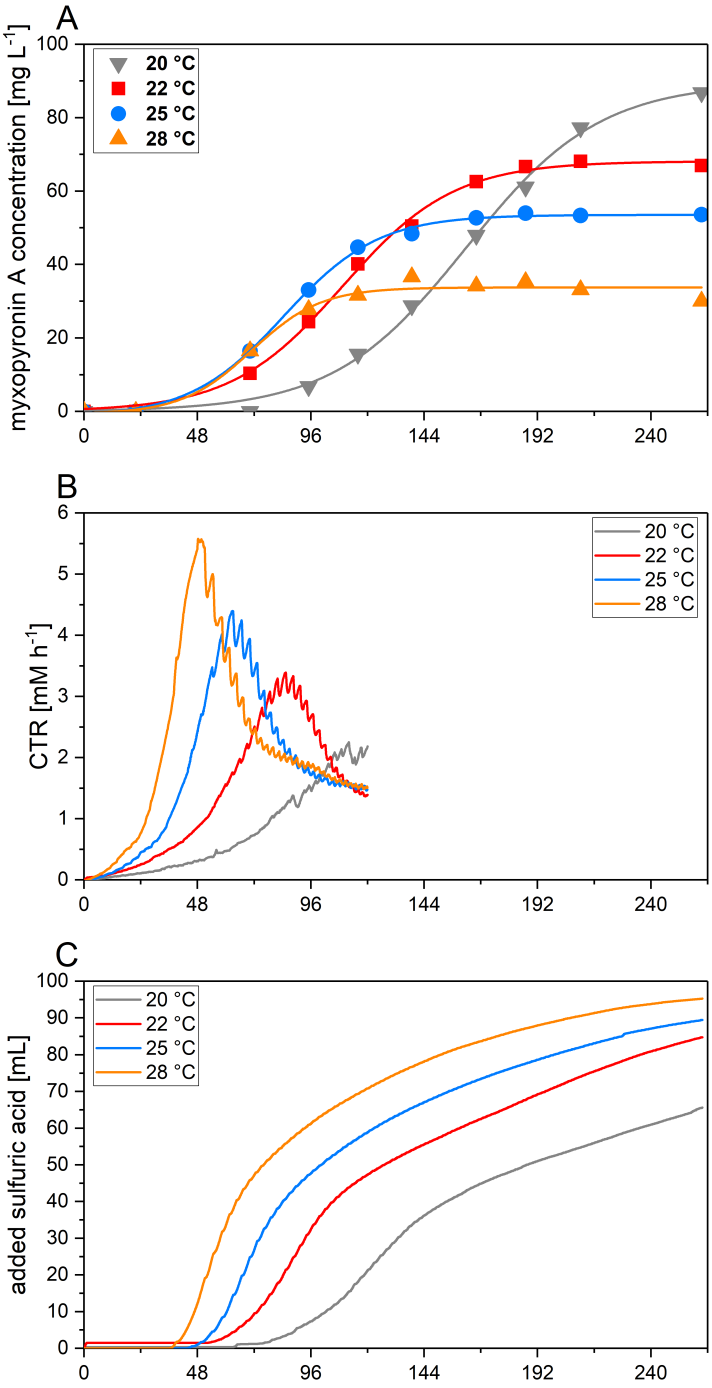

Figure 3.8: Course of **A** the myxopyronin A concentration, **B** the carbon dioxide transfer rate (CTR) and **C** added sulfuric acid over the cultivation time for bioreactor fermentations with standard medium at different temperatures.

To combine the results of the media screen and those of the cultivations at different temperatures, bioreactor fermentations with meat peptone medium, the substrate with which the highest product concentrations could be obtained in the media screening, were ensued. As the product formation in the

cultivation at 20 °C was much slower than this at 22 °C (stationary phase reached after 166 h compared to 261 h at 20 °C), cultivations with the meat peptone medium were only done at 22 °C, 25 °C, 28 °C and 30 °C. The results are shown in Figure 3.9.

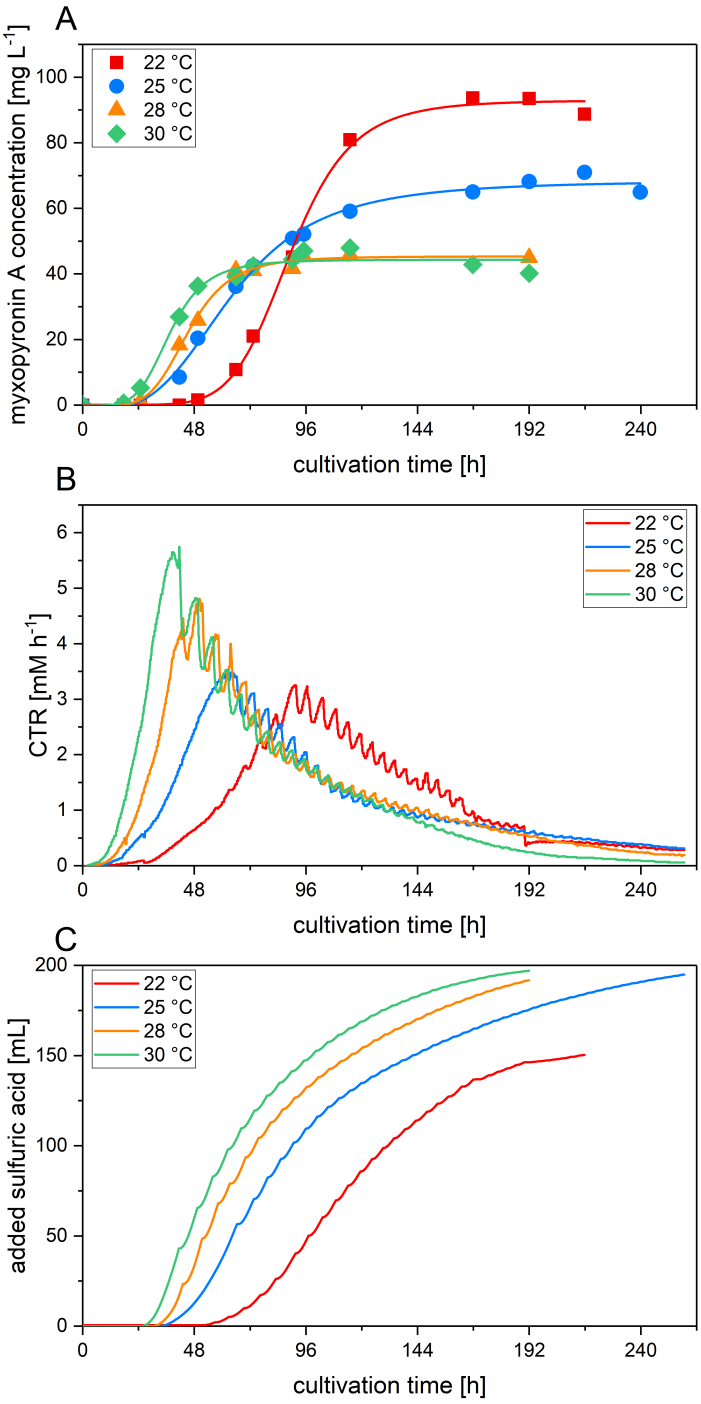

Figure 3.9: Course of **A** the myxopyronin A concentration, **B** the carbon dioxide transfer rates (CTR) and **C** the added volume of sulfuric acid over the cultivation time for bioreactor fermentations with meat peptone medium at different temperatures.

In accordance to the cultivations done with the standard medium, temperature also has a negative influence on the myxopyronin A concentrations when using the meat peptone medium. The highest concentration was reached at 22 °C (93.7 mg L^{-1}), whereas at 25 °C, 28 °C and 30 °C maximum concentrations of 71.0 mg L^{-1}, 46.0 mg L^{-1} and 47.9 mg L^{-1}, respectively, could be achieved. Notably, the product concentrations as well as the product formation kinetics were similar at 28 °C and 30 °C with the meat peptone medium, which could not be observed with the standard medium. In general, the product titers in meat peptone medium were higher at all temperatures than the cultivations done with the standard medium. In comparison to the starting conditions (30 °C and skim milk medium) the product concentration could be increased 5.6-fold when using meat peptone medium and a cultivation temperature of 22 °C.

When looking at the CTR, the growth of the culture again correlated with the product formation. The latter started for all cultivations in the late exponential growth phase. In the cultures with 28 °C and 30 °C cultivation temperature, product formation soon stagnated whereas at 22 °C and 25 °C the myxopyronin A concentration still increased during the stationary growth phase. The oscillation of the CTR is caused by the fact that whenever the culture becomes more acidic, carbon dioxide that was formerly dissolved in the medium is released. When the pH is regulated by adding potassium hydroxide more carbon dioxide can be dissolved again. Therefore, these fluctuations do not represent the true carbon dioxide emission rate (CER), a problem that has been addressed by several publications before [219, 220, 218]. The added volume of sulfuric acid also correlated with the CTR. Notably, the CTR does not differ significantly between the fermentations in the standard (skim milk powder) and meat peptone medium, whereas the added volume of sulfuric acid is about factor two higher in the meat peptone medium. Thus, this parameter can only be compared for cultivations in the same medium.

In an effort to increase the product concentration further, the possible bottleneck of building block supply was addressed. The eastern chain of myxopy-

ronin A contains one glycine and three malonyl-CoAs whereas the western chain is made up out of acetyl-CoA as a starter unit, elongated by three malonyl-CoAs [138]. Malonyl-CoA is synthesized from acetate via acetyl-CoA [161]. Thus, acetate supply was increased by using acetic acid for pH correction instead of sulfuric acid. In a second fermentation, the effect of kanamycin on the product formation was explored. Kanamycin was thus far used to keep up the selective pressure to ensure the incorporation of the heterologous gene cluster. Furthermore, it could be deduced from the experiments presented in Figure 3.9 that myxopyronin A formation correlated with the growth of the culture. In order to prolong the growth phase and correspondingly the time of product formation, a fed-batch fermentation was performed. The feed consisted of meat peptone medium with 3-times concentrated meat peptone and was started 96 h after inoculation. It was kept at constant speed for another 96 h. The product concentrations, CTRs and added acid volume of these fermentations are presented in Figure 3.10 as well as those of the standard fermentation at 22 °C for comparison.

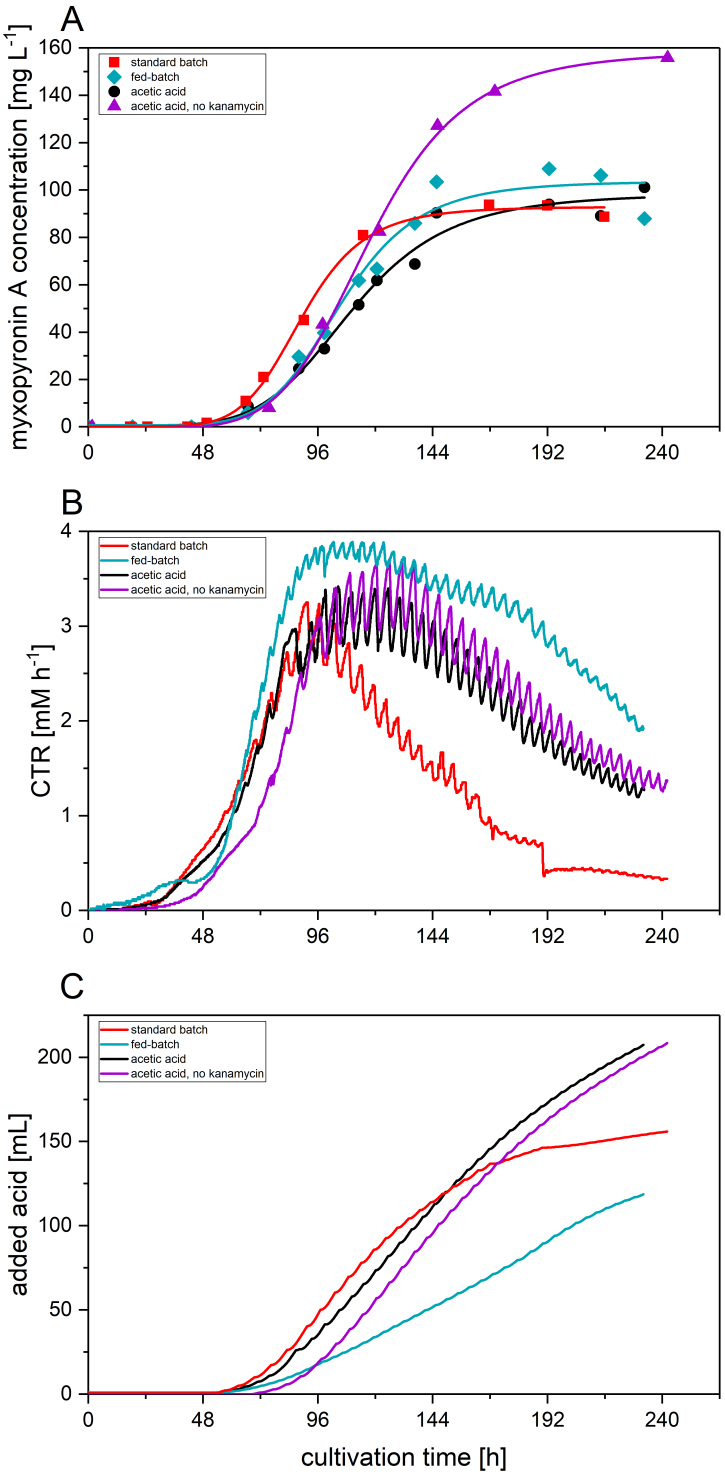

Figure 3.10: **A** Myxopyronin A concentrations, **B** carbon dioxide transfer rates (CTR) and **C** added acid volume over the course of the cultivation time for the standard batch at 22 °C, a fed-batch (with sulfuric acid for pH correction) and the fermentations using acetic acid for pH correction and without kanamycin (also at 22 °C).

When looking at the myxopyronin A concentration, it can be deduced that using acetic acid for pH correction only has a slight positive effect that re-

sults in a maximum concentration of 101.1 mg L^{-1}. Nevertheless, it seemed to be beneficial for the bacterial growth as the CTR did not decline as fast as the one of the standard fermentation. For this reason, acetic acid was also used when testing the influence of kanamycin on the product formation. The CTRs of the fermentations with and without kanamycin are similar, but the product concentration is significantly higher without kanamycin with a maximum myxopyronin A concentration of 142.2 mg L^{-1}. The volume of added acid was also higher in the cultivations with acetic acid compared to the standard batch. The fed-batch fermentation resulted in only slightly increased product concentrations (109.0 mg L^{-1}) although the CTR was higher than this of the standard batch. The volume of added acid on the other hand was 24 % lower.

In general, the product concentration could be increased from 16.9 mg L^{-1} (skim milk medium at 30 °C with sulfuric acid and kanamycin) to 142.2 mg L^{-1} (meat peptone medium at 22 °C with acetic acid without kanamycin) which accounts for an almost tenfold increase only through adjustment of media components and cultivation parameters.

3.2.2 Heterologous Production of Vioprolides

Natural product formation of myxobacteria largely depends on the choice of substrate in nutritional media because of their complex nutritional requirements. Naturally, *Myxococcus xanthus* feeds on other bacteria, a complexity of nutrients which is hard to mimick in cultivation media. In an attempt to find a suitable substrate, different complex carbon-nitrogen-sources (CN-sources) were tested for their ability to promote vioprolide production. The CN-sources used were skim milk powder, casein, casitone, meat peptone, yeast extract and soy peptone, media components frequently used for cultivation of *M. xanthus* [191, 193]. The medium containing 20 g L^{-1} skim milk was set as a basis. Via HPLC measurements, it could be deduced that skim milk powder consists of 30 % lactose (data not shown). Thus, in the media containing the other CN-sources, the 20 g L^{-1} skim milk powder was substi-

tuted by 14 g L^{-1} CN-source and 6 g L^{-1} lactose to avoid any bias through omitting the sugar. The results of the shake flask experiment for castione, yeast extract, meat peptone and soy peptone can be seen in Figure 3.11. It was not possible to determine the product concentrations for skim milk powder and casein as a substrate, because the product peaks were overlaid by media component peaks.

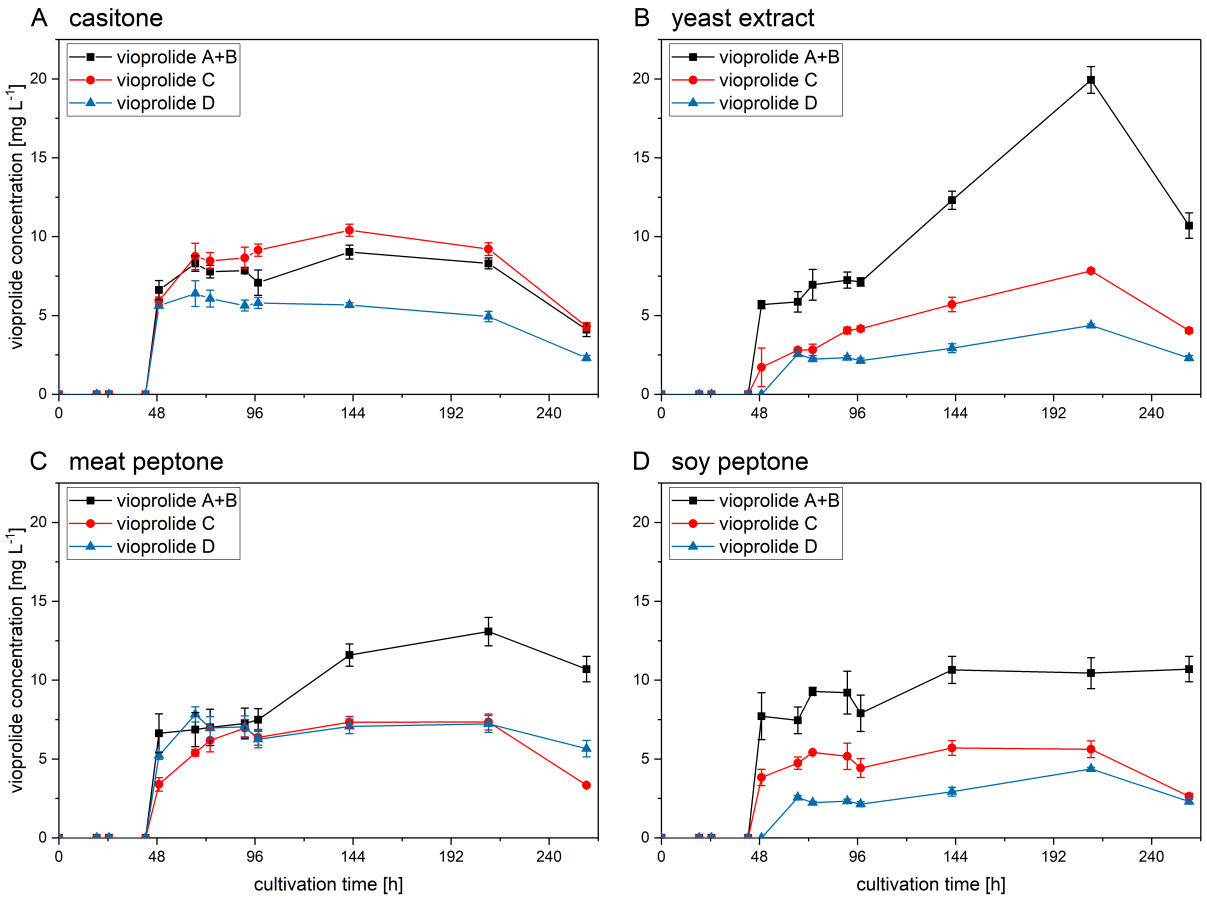

Figure 3.11: Concentrations of the different vioprolide derivatives over the course of the shake flask cultivation for **A** casitone, **B** yeast extract, **C** meat peptone and **D** soy peptone. Data points are the mean value of three shake flasks, the error bars represent the standard deviation.

As can be seen, vioprolides A and B are determined together as it was impossible to separate the two peaks with the analytical HPLC columns available. Schummer *et al.* could separate the two derivatives only with reversed-phase and subsequent normal-phase chromatography [77]. As this procedure was too time-consuming considering the number of samples, the two peaks were

detected together.

When comparing the different CN-sources, it can be seen that product formation started at 48 h with maximum concentrations between 4.4 mg L^{-1} and 19.9 mg L^{-1} with yeast extract yielding the highest concentration of vioprolides A+B and meat peptone of vioprolide C. Meat peptone was furthermore the only substrate where vioprolide C was the most abundant derivative. Based on these results, bioreactor cultivations were conducted to verify the product formation, the results of which are presented in Figure 3.12.

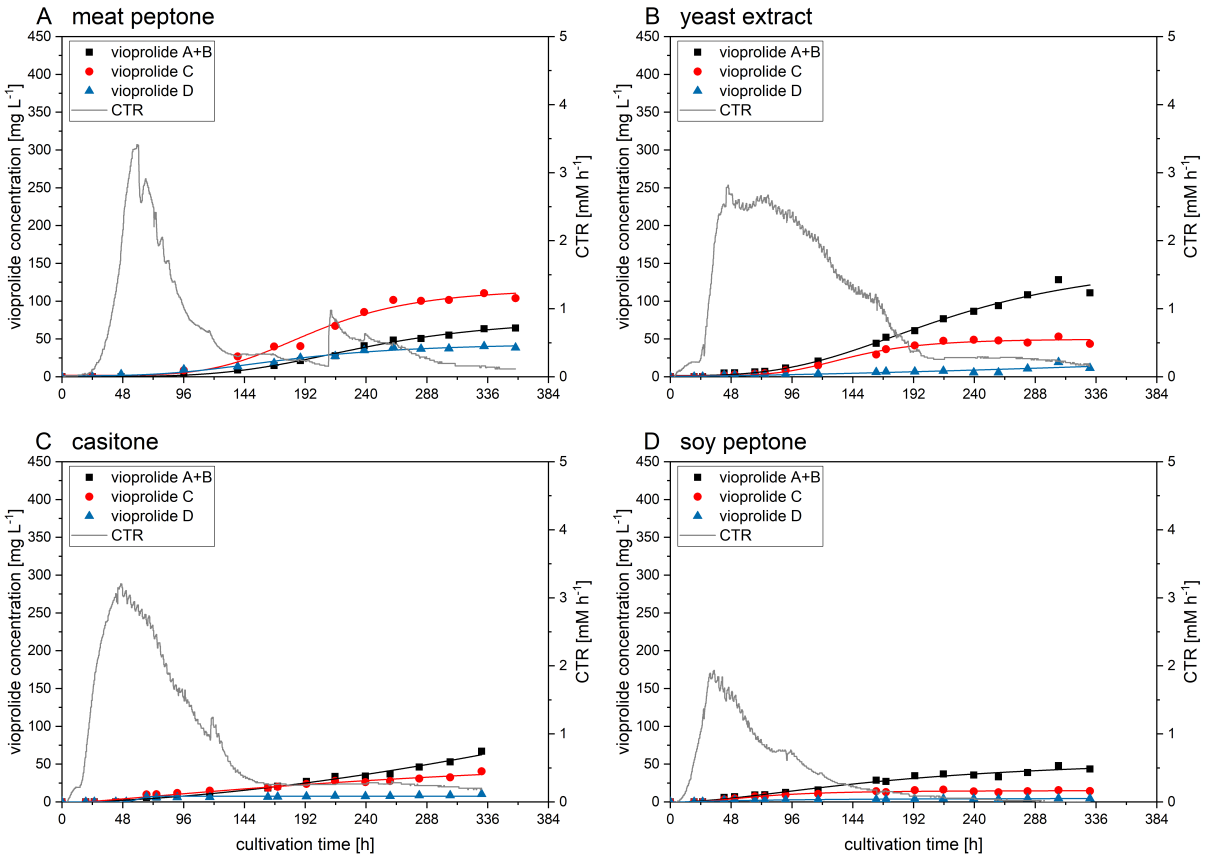

Figure 3.12: Concentrations of the different vioprolide derivatives over the course of the bioreactor cultivation for **A** meat peptone, **B** yeast extract, **C** casitone and **D** soy peptone. The gray line represents the carbon dioxide transfer rate (CTR).

The product concentrations for the bioreactor cultivations were higher than in the shake flask experiments, with concentrations of up to 128.4 mg L^{-1} for vioprolides A+B (in yeast extract). These high titers were especially reached when using meat peptone and yeast extract. But these two substrates differed in the most abundant derivative (vioprolide C for meat peptone, vioprolides

A+B for yeast extract) and in the growth characteristics of the culture which can be deduced by the carbon dioxide transfer rate (CTR). For meat peptone, the highest CTR value (3.4 mM h^{-1}) was reached after 59.5 h with a steady decline afterwards. After 144 h, only minimal respiratory activity could be monitored. For yeast extract, the highest CTR value of 2.8 mM h^{-1} was lower than when using meat peptone, but it was already reached after 45.5 h.

Furthermore, the decline of the CTR was slower with minimal respiratory activity detected after 192 h. This shows that meat peptone was metabolized more rapidly, but was already depleted after 59.5 h, whereas yeast extract was metabolized more slowly. The oscillation of the CTR is caused by the fact that whenever the culture becomes more acidic, carbon dioxide that was formerly dissolved in the medium is released. When the pH is regulated by adding potassium hydroxide more carbon dioxide can be dissolved again. Therefore, these fluctuations do not represent the true carbon dioxide emission rate (CER), a problem that has been addressed by several publications before [218, 219, 220]. The formation of vioprolides started when the CTR is already declining, in the so-called stationary phase, which is typical for secondary metabolites [221]. Since casitone and soy peptone yielded only maximum concentrations of 67.0 mg L^{-1} and 48.0 mg L^{-1} vioprolides A+B, respectively, they were not considered for further experiments.

In a consecutive attempt to reach the goal of improved vioprolide yields, shake flask cultivations were performed where amino acids that are vioprolide buliding blocks were fed to cultures growing in yeast extract medium. The results were striking (Figure 3.13). As in the previous cultivations with yeast extract medium, vioprolides A+B were the most abundant derivatives. Interestingly, the concentration of vioprolides A+B could be enhanced even more by adding DL-pipecolic acid to the culture (43.0 mg L^{-1} compared to 30.9 mg L^{-1} for the control). On the other hand, the concentration was lower when L-proline was added (20.8 mg L^{-1}). The contrary can be seen when looking at vioprolides C and D. Here, the product concentrations were

higher when L-proline was added with 13.4 mg L^{-1} and 14.1 mg L^{-1} for vioprolides C and D, respectively, than for the control (11.6 mg L^{-1} vioprolide C and 7.6 mg L^{-1} vioprolide D). The addition of DL-pipecolic acid led to decreased concentrations of vioprolide C and D, with 3.3 mg L^{-1} and 3.0 mg L^{-1}, respectively.

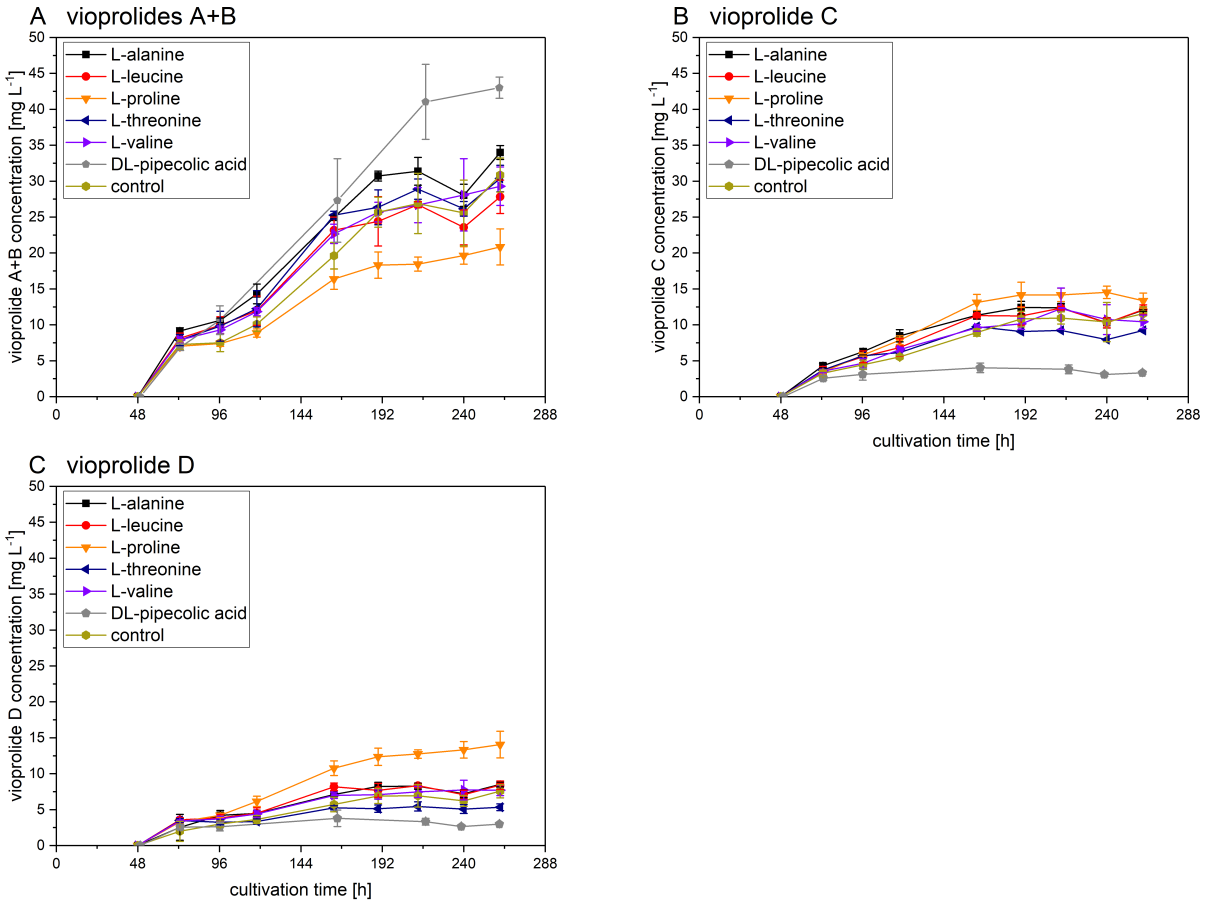

Figure 3.13: Concentrations of **A** vioprolides A+B, **B** vioprolide C and **C** vioprolide D after addition of different amino acids to the shake flask cultures growing in yeast extract medium. The amino acids were added after 48 h. Data points are the mean value of three shake flasks, the error bars represent the standard deviation.

Bioreactor cultivations were performed in order to verify the results obtained from the amino acid feeding experiments. Either L-proline or DL-pipecolic acid was added to cultures in meat petone and yeast extract medium. Strikingly, a maximum concentration of 141.4 mg L^{-1} (Figure 3.14D) was reached which is only 13.0 mg L^{-1} higher than this obtained without any amino acid supplementation (Fig. 3.12B). In the shake flask experiment, the titer of

vioprolides A+B could be increased 1.4-fold through the addition of DL-pipecolic acid (Fig. 3.13A). Although the concentration of vioprolides A+B could not be enhanced, the formation of the other two derivatives could apparently be suppressed in this experiment. Only 24.6 mg L^{-1} vioprolide C and 14.3 mg L^{-1} vioprolide D could be detected in comparison to 53.2 mg L^{-1} and 19.2 mg L^{-1} in the fermentation without any amino acids. The addition of L-proline to the culture in yeast extract medium caused a shift from vioprolidea A+B to vioprolide C being the most abundant derivatives. The maximum concentration of vioprolide C was 128.8 mg L^{-1} (Fig. 3.14C) which accounted for a 2.4-fold increase compared to plain yeast extract medium (53.2 mg L^{-1}) (Fig. 3.12B). Furthermore, the yield of vioprolide D could be enhanced as well from 19.2 mg L^{-1} to 36.4 mg L^{-1}.

The results obtained from adding DL-pipecolic acid or L-proline to cultures with meat peptone medium completed the picture. Through supplementation of L-proline, the concentration of vioprolide C could be increased from 110.5 mg L^{-1} (Fig. 3.12A) to 182.2 mg L^{-1} (Fig. 3.14A). The vioprolide D titer was also positively affected by the addition of L-proline (50.8 mg L^{-1} compared to 40.5 mg L^{-1}). Even more striking is the result of the supplementation of DL-pipecolic acid to the culture in meat peptone medium. Here, the vioprolides A+B concentration could be increased 6.2-fold from 64.5 mg L^{-1} (Fig. 3.12A) to 400.0 mg L^{-1} (Fig. 3.14B).

Subsequently, bioreactor cultivations with combined supplementation of L-proline and DL-pipecolic acid to meat peptone and yeast extract medium were performed. This led to the highest concentration of vioprolide C obtained in yeast extract medium with a maximum of 179.8 mg L^{-1} (Fig. 3.15B). Although DL-pipecolic acid was fed as well, the titer of vioprolide A+B was lower (71.2 mg L^{-1}) than when feeding only L-proline (116.6 mg L^{-1}) (Fig. 3.14C). The vioprolide D concentrations stayed the same with a maximum concentration of 34.4 mg L^{-1} compared to 36.4 mg L^{-1} when only L-proline was fed.

The combined supplementation of L-proline and DL-pipecolic acid to the cul-

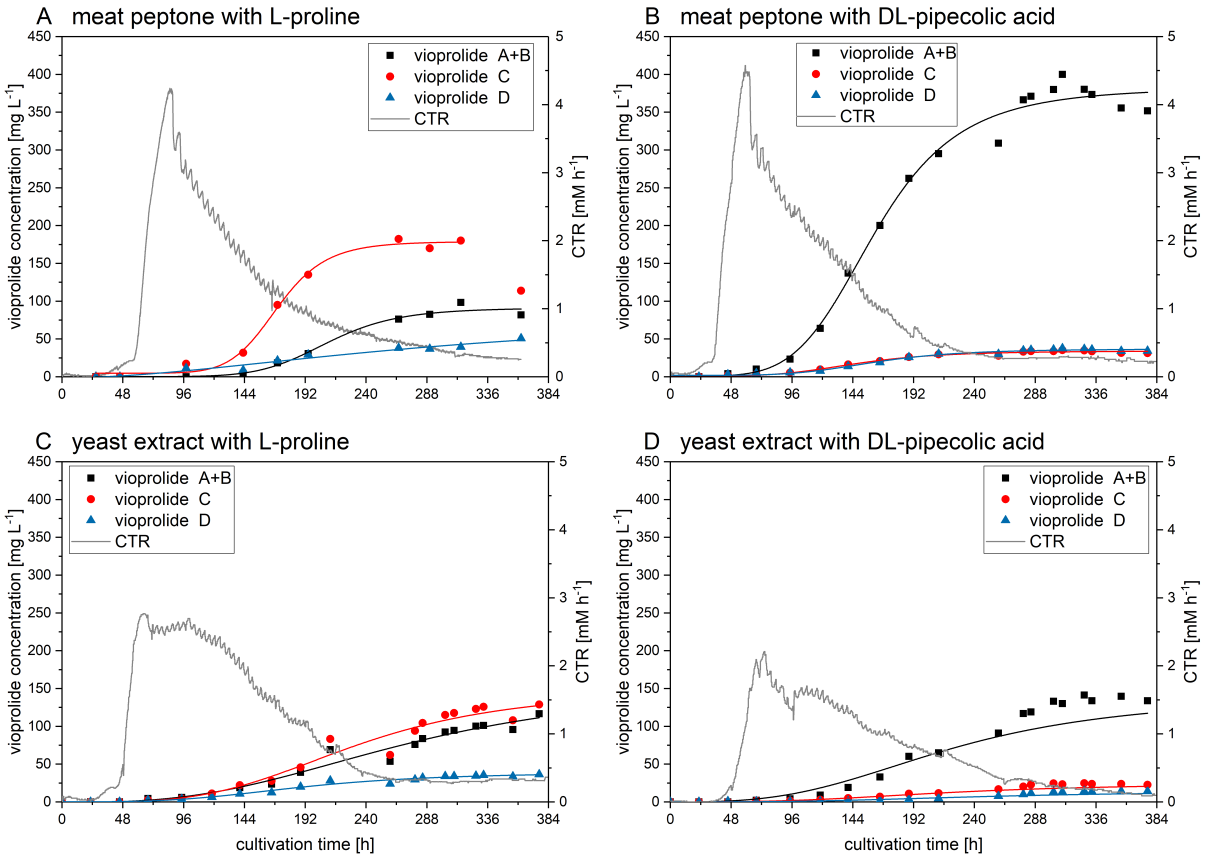

Figure 3.14: Concentrations of the different vioprolide derivatives over the course of the bioreactor cultivation for **A** meat peptone with L-proline, **B** meat peptone with DL-pipecolic acid, **C** yeast extract with L-proline and **D** yeast extract with DL-pipecolic acid. The gray line represents the carbon dioxide transfer rate (CTR). The amino acids were added after 48 h.

ture grown in meat peptone medium did not have any positive effect on the vioprolide yield. The derivatives A+B were the most abundant ones with a maximum concentration of 168.0 mg L^{-1}, as through supplementation with solely DL-pipecolic acid, but without reaching its titer (Fig. 3.15A). The concentrations of vioprolides C and D were at 52.0 mg L^{-1} and 41.5 mg L^{-1}, respectively.

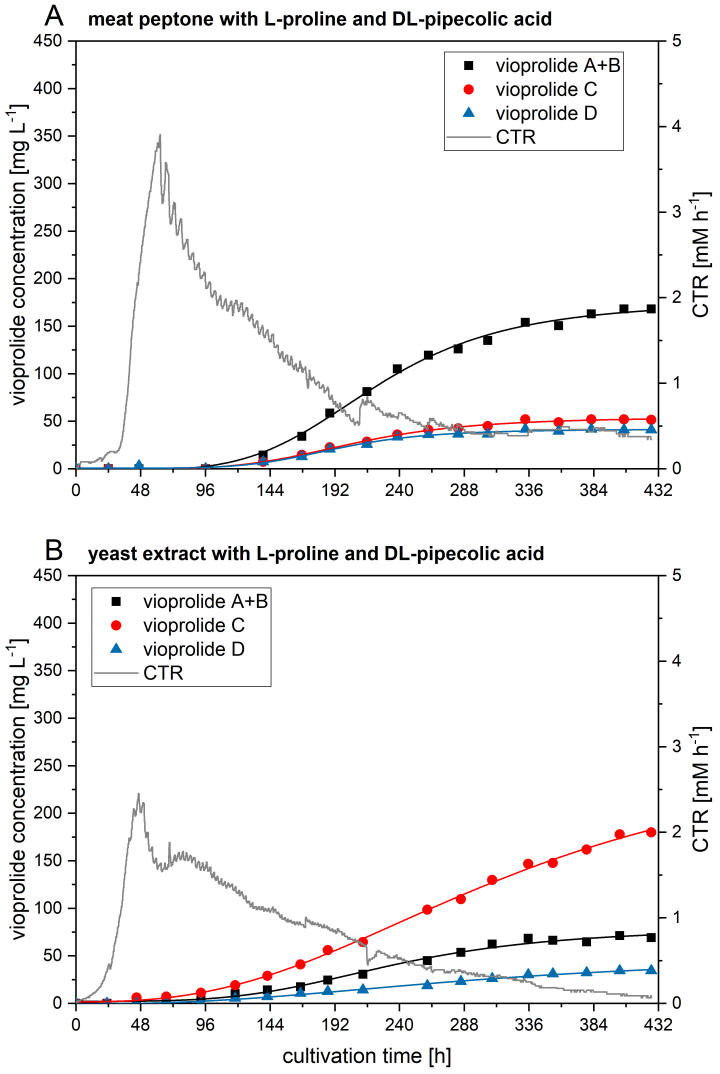

Figure 3.15: Concentrations of the different vioprolide derivatives over the course of the bioreactor cultivation for **A** meat peptone with L-proline and DL-pipecolic acid and **B** yeast extract with L-proline and DL-pipecolic acid. The gray line represents the carbon dioxide transfer rate (CTR). The amino acids were added after 48 h.

4 Discussion

4.1 Production of Elansolids with *Chitinophaga sancti* Fx7914

Elansolids are interesting candidates for drug development, but chemical synthesis is to date only available for elansolid B1 [222] and not feasible in multi-gram scale. Therefore, provision of substantial amounts of elansolids to enable further semi-synthetic drug development is only achievable by biotechnological means. The chosen strategy was two-fold: Our first approach aimed towards the production of elansolid A2. This is an ideal start-

ing point for chemical optimization of the molecule where the ring-structure shall be retained whereas our second approach aimed towards the biotechnological production of a linearized derivative by adding a nucleophile (in our case anthranilic acid) to the culture, thereby preventing the accumulation of undesired side products.

In the batch fermentation, we could show that sucrose was cleaved and the monosaccharides were metabolized separately, with glucose as the preferred substrate, yielding 18.9 mg L^{-1} elansolid A2. The cleavage of sucrose might be carried out by extracellular invertases. These findings are well in accordance with publications showing that *Chitinophaga pinensis*, a close relative of *C. sancti*, possesses a variety of carbohydrate-active enzymes (CAZymes) for the degradation of carbohydrates, among them invertase [87, 223, 224, 225]. The preferred usage of glucose over other carbon sources has been well observed in different types of organisms under the term carbon catabolite repression [226, 227].

To increase elansolid A2 production, a fed-batch fermentation was applied. Sucrose was chosen as the carbon source in the basal medium whereas glucose was provided in the feed solution as it was shown to enable rapid growth. Hence, the product concentration could be increased almost 3-fold from 18.8 mg L^{-1} in the batch fermentation to 55.3 mg L^{-1} in the fed-batch fermentation. The fed-batch fermentation with glucose as sole carbon source resulted in only 31.8 mg L^{-1} elansolid A2 but 223.1 mg L^{-1} elansolid C1. Elansolid C1 is generated when anthranilic acid acts as a nucleophile that attacks the quinone methide ring of elansolid A3 [120]. Since in this process anthranilic acid must be produced by the organism itself presumably as a precursor for tryptophan biosynthesis, accumulation of anthranilic acid and its subsequent side reactions with elansolid A3 could possibly be prevented by feeding tryptophan and thereby exploiting a possible feedback inhibition of the trp-operon [228]. This will be subject of further research for process improvement. Furthermore, the feeding rates need to be adapted to avoid glucose accumulation during the feeding phase. In two of the fed-batch

fermentations, glucose accumulated to final concentrations of 37.3 mg L^{-1} and 77.5 mg L^{-1} and although growth and product formation did not seem to be affected by a potential overflow metabolism, the accumulation of not metabolized substrates reduces process profitability and should therefore be minimized in future experiments.

To prove the concept of precursor-directed biosynthesis of novel elansolid derivatives that harbor several interesting advantages, anthranilic acid was provided in the medium as a precursor in another fermentation. Here, an elansolid C1 concentration of 257.5 mg L^{-1} has been obtained. This yield is equivalent to a 36-fold increase in product concentration compared to 7.1 mg L^{-1} which could be achieved by fermentation so far.

The precursor-directed biosynthesis during fermentation when providing anthranilic acid allowed the formation of the desired product in high amounts whereas formation of other elansolid derivatives occurred only in minor concentrations (85 % elansolid C1, Figure 3.4C). Accordingly, this procedure should be further investigated as basis for the creation of different novel elansolid derivatives, since the semi-synthetic approach within crude extracts proposed by Steinmetz *et al.* [120] is accompanied by a considerable loss of product.

The precursor-directed elansolid production is of special interest since although the absolute amount of elansolid A2 was increased considerably comparing absolute product titers of batch fermentation and fed-batch fermentation with sucrose, its share of the overall elansolid yield was similar with 14 % in the batch (Figure 3.1c) and 15 % in the fed-batch process (Figure 3.2c). The presence of these side-products increases the workload for purification of elansolid A2. Subsequently, after chromatographic purification of elansolid A2, which is additionally hampered by the stability of the molecule, the compound must be chemically converted into the desired product. This can only be accomplished with considerable losses at this time [119].

In this light, the precursor-directed synthesis of new elansolid derivatives directly during the fermentation might provide a promising tool in the search

for novel antibiotics, especially since conversion into the more stable non-cyclic derivative elansolid C1 is accompanied by only a minor loss of activity even when observing Gram-negative permeabilized cells. Thus, one focus of medicinal chemistry approaches could be the structure modification towards increased cell wall penetration.

4.2 Heterologous Production of Myxobacterial Products with *Myxococcus xanthus* DK1622

4.2.1 Heterologous Production of Myxopyronin A

Complex media components are essential for the growth of *M. xanthus* DK1622, as they provide carbon and nitrogen in form of proteins and peptides. Skim milk powder consists of approximately 36 % proteins, of which casein makes up about 80 % [229]. Consequently, the composition of the skim milk (SM) and casein (CS) media were similar in terms of protein content. Since the myxopyronin A concentrations were also similar in both media, the substitution of skim milk with lactose and casein seemed to have no influence. Slightly lower product concentrations could be obtained with casitone, a pancreatic digest of casein, which contains peptides in different size (up to 5,000 Da) [230]. Casamino acids are acidic hydrolysates of casein, which results in single amino acids and small peptides (90 % $<$ 250 Da) [231]. When comparing the product titer and the growth curve of the different milk protein media, it can be deduced that the more the proteins are broken up into smaller peptides or single amino acids the lower the product concentration. This phenomenon has already been observed before [191]. Yeast extract contains peptides with higher molecular weight (up to 5,000 Da), but also contains carbohydrates (approx. 16 %). The same holds true for soy peptone (approx. 30 % carbohydrates) [232]. As carbohydrates cannot be metabolized by *M. xanthus*, the overall nutritional value is lower than this of pure protein hydrolysates. The highest myxopyronin A concentration however was observed with the meat peptone medium. This can be explained by the presence of peptides with higher molecular weight (up to 10,000 Da) [233].

Besides media composition, process parameters can have an influence on the growth of the culture and concomitantly on the product concentration. Therefore, the influence of temperature, pH, DO setpoint and air flow rate was investigated. The pH can have an influence on the growth of the organism as well as the stability of the product in the culture medium. Hüttel and Müller could also show that the product profile of *Chondromyces crocatus*, another myxobacterium, changed depending on O_2 and CO_2 levels ([197]. The level of dissolved CO_2 in a medium is smaller at higher air flow rates because of the faster gas exchange. Different product profiles could also be observed for the heterologous expression of epothilones in *M. xanthus* DZ1 [196]. However, in this study, solely the temperature had a significant influence on the myxopyronin A concentration. In case of the soraphens, temperatures over 30 °C drastically decreased the product concentrations as also observed in this study [56]. Several studies have also shown that a lower temperature aids in the correct folding of the biosynthetic PKS machinery when heterologously expressed in *E. coli* [161, 163, 234]. Another reason could be a redistribution of nutritional resources to secondary metabolite production when growth is forced to be slowed down due to lower cultivation temperatures. The Design of Experiments setup helped to rule out any interactions of two factors that would not have been recognized when employing the one-factor-at-a-time approach. The benefits of a statistical approach on all fields of process optimization are also summarized in an excellent review [200].

After exploring the growth and product formation kinetics at different temperatures, 22 °C was chosen as cultivation temperature for further experiments. Here, the addition of acetic acid for pH correction showed to be beneficial for the growth of the culture resulting in a slightly higher CTR combined with a slower decline at the end of the fermentation. This could be explained by the fact that *M. xanthus* is able to use some organic acids, among them acetate, as substrate [192, 235].

The fed-batch fermentation did not yield significantly higher product con-

centrations than the standard batch, although the CTR was increased. One reason could be that the concentration of meat peptone was too high in this regard that its catabolism resulted in an accumulation of ammonia that was inhibitory for the organism. Normally, this is also reflected in an increased addition of acid. In this case, this logic was subverted by the addition of the feed that was slightly acidic after autoclaving. Future experiments will involve optimization of feed composition and profile to be able to increase the final product concentrations.

Omitting kanamycin during cultivation increased the myxopyronin A concentration by 40 mg L^{-1}. Although chromosomal integration of gene clusters is regarded as stable, kanamycin was used because of the ability to study the behavior of the organism without the possibility of losing the gene cluster. But the integration seemed to be stable as even more product was built. This could be due to the lower metabolic burden, because resistance enzymes did not be expressed if no kanamycin is present in the culture.

In general, it could be shown that heterologous expression of myxobacterial natural products in the closely related host *Myxococcus xanthus* DK1622 can be a mean to increase the product supply for further research. In this study, the production of one of two myxobacterial α-pyrone antibiotics was investigated. As the biosynthetic gene cluster of the other one, namely corallopyronin, has also been integrated into *M. xanthus* DK1622 recently [214], it will be interesting to see if the results obtained for myxopyronin can be applied to corallopyronin production as well. This could be a major step on the way of promoting *M. xanthus* DK1622 as a high-titer expression platform for myxobacterial natural products.

4.2.2 Heterologous Production of Vioprolides

The goal of this study was to find a suitable production process for the vioprolides originally produced by *Cystobacter violaceus* Cb vi35. As Yan *et al.* established, the production level was significantly higher in the heterologous producer *M. xanthus*::Ptn5-vio than in the original producer [215]. However,

the titers that could be reached in the first shake flask experiments were only a total of 35.0 mg L^{-1} vioprolides in yeast extract medium and a total of 24.8 mg L^{-1} in meat peptone medium (Figure 4.1).

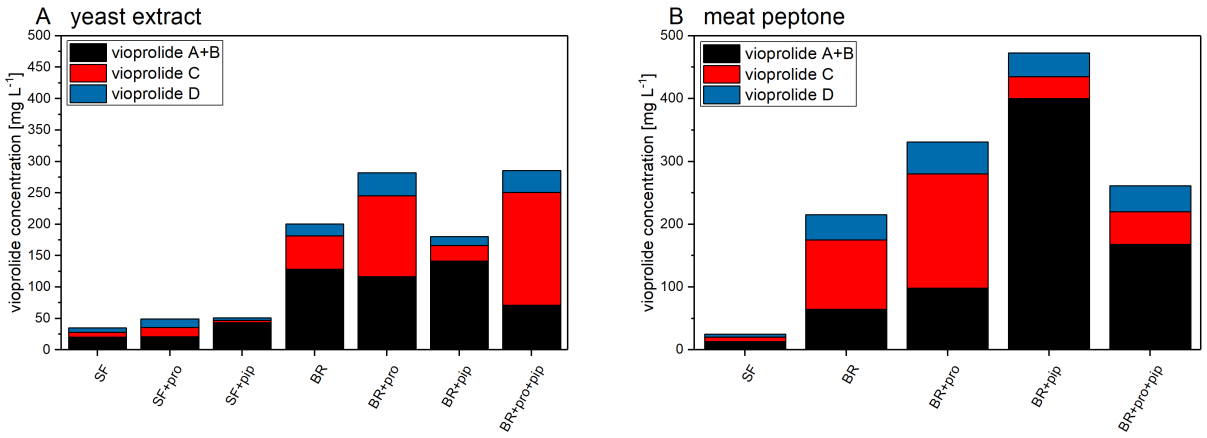

Figure 4.1: Summed concentrations of all vioprolide derivatives for the experiments carried out in **A** yeast extract medium and **B** meat peptone medium. SF=shake flask, BR=bioreactor, pro=L-proline, pip=DL-pipecolic acid.

An increase of the product titers could be achieved through bioreactor cultivations. Here, concentrations of 200.8 mg L^{-1} vioprolides A-D (for yeast extract medium) and 215.5 mg L^{-1} (for meat peptone medium) could be reached. This difference originates most likely from the cultivation environment that is more controlled in the bioreactor cultivations than in the shake flask experiments.

One parameter that is regulated in bioreactor cultivations but not in shake flaks experiments is the pH. As can be seen in Figure 4.2, the pH increased despite the addition of HEPES to the medium from 7.2 at the beginning of the cultivation to a value of 8.6 for meat peptone medium (after 210.5 h) and 8.7 for yeast extract medium (after 142.3 h).

The pH would have also increased in the bioreactor cultivations, if it had not been kept at a constant level through the addition of sulfuric acid (Figure 4.3). The increased addition of sulfuric acid to the cultivation in meat peptone medium (133 mL) can be correlated to the higher CTR (Fig. 3.12A) compared to the cultivation in yeast extract medium (Fig. 3.12B). This fact can be explained by a somehow higher nutritional value of the meat peptone

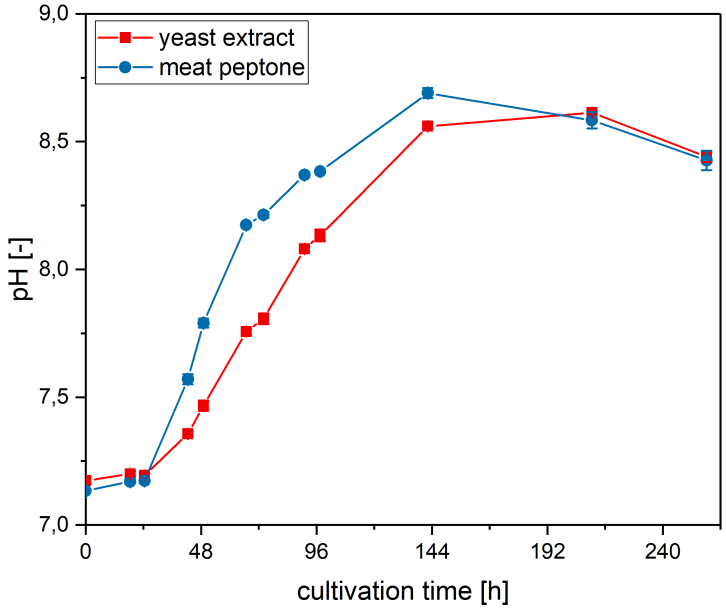

Figure 4.2: Course of the pH over the cultivation time for shake flask experiments carried out in meat peptone and yeast extract medium. Data points are the mean value of three shake flasks, the error bars represent the standard deviation.

medium that allowed a faster metabolism and therefore faster growth. The end product of amino acid metabolism (the components of peptones) is ammonia [193, 235]. It is excreted which ultimately leads to an increase in the pH that in turn is counteracted by the addition of sulfuric acid. The more amino acids are metabolized the more ammonia is excreted and the more sulfuric acid needs to be added to keep the pH constant.

In order to test whether the higher product concentrations in the bioreactor cultivations could be attributed to the constant pH, a bioreactor cultivation with HEPES but without the addition of sulfuric acid was performed (Fig. 4.4). As can be seen, the pH also increased from 7.5 to a value of 8.8 at the end of the cultivation (Fig. 4.4B). The CTR is similar between the cultivations with and without the addition of HEPES. With a total of 64.0 mg L^{-1} vioprolides the concentrations lay between that of the shake flask experiments and the bioreactor cultivations. Therefore, the constant pH in the bioreactor cultivations possibly has a positive influence on the product titer. One explanation could be a decreased stability of the vioprolides at high pH. But there are also other cultivation parameters that cannot be controlled in shake flask

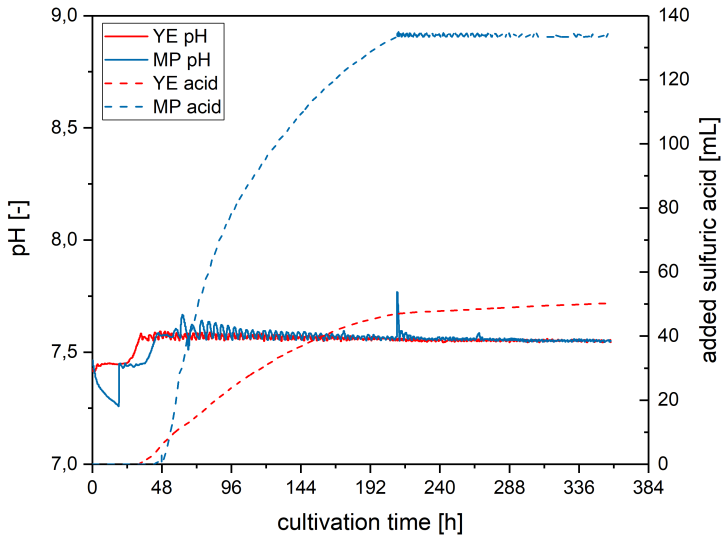

Figure 4.3: Course of the pH and the added volume of sulfuric acid over the cultivation time for bioreactor cultivations performed in meat peptone and yeast extract medium. YE=yeast extract, MP=meat peptone.

experiments which could have an influence on the product titer, like the oxygen supply. As Hüttel & Müller could show, the secondary metabolite profile of a *Chondromyces* strain varied between bioreactor cultivations with different DO setpoints [197]. More studies have to be conducted in order to find the reason for the increased vioprolide production in bioreactor cultivations, also with the prospect of optimizing these even further.

One observation that could be made in the shake flask experiments was the control of the derivative distribution through the supplementation of specific amino acids. These results could also be verified in the bioreactor cultivations (Fig. 4.1). Addition of DL-pipecolic acid led to increased titers of vioprolides A+B in yeast extract as well as in meat peptone medium. L-pipecolic acid is a six-ring building block of vioprolides A and B and a non-proteinogenic amino acid. Although the racemic mixture was used, it can be assumed that only the L-isomer was incorporated as previously observed [170, 171]. This could be investigated further by feeding only the pure isomers. This would also increase the vioprolide yield relating to the amount of used pipecolic acid. As racemically pure substance is quite expensive, another way of supplying L-pipecolic acid could be the feeding of L-lysine. In bacteria, pipecolic acid was found to be generated from lysine through different reactions [236].

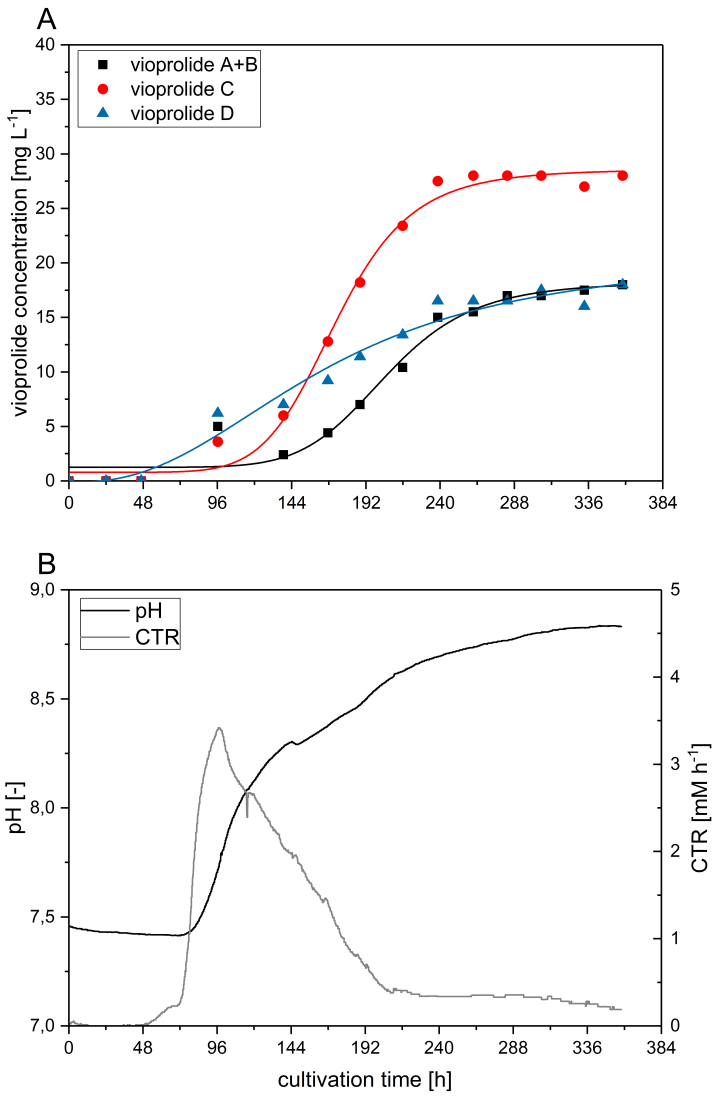

Figure 4.4: **A** Concentrations of the different vioprolide derivatives and **B** the course of the pH and the CTR over the course of the bioreactor cultivation in meat peptone medium with HEPES.

Chai *et al.* could elucidate that a lysine cylcodeaminase catalyzes this reaction step in the myxobacterium *Angiococccus disciformis* An d48, where piepcolic acid serves as a starter unit for tubulysin biosynthesis [170]. This enzyme is also involved in provision of pipecolic acid for other PK/NRP-based natural products [237, 238]. If, on the other hand, this cyclodeamination step is the bottle neck in pipecolic acid supply, the feeding of L-lysine would not enhance the titers of vioprolide A+B. This conclusion was made after feeding DL-piecolic acid to a culture of a heterologous *Pseudomonas putida* strain producing tubulysin. The supplementation also led to substantial increases in pre-tubulysin A [171].

Vioprolides A and C are one of the few examples of natural products containing an azetidine ring. A few plant-derived compounds like mugineic acid [239], calydaphninone [240], gelsemoxonine [241] and azetidine-2-carboxylic acid are known of which biosynthesis studies showed that azetidine is formed from 2,4-diaminobutyric acid which itself derives from S-adenosyl-L-methionine or homoserine [242]. Azetidine-2-carboxylic acid which only lacks the methyl group at C_4 in comparison to 4-Maz was discovered in lilies, poincianas and sugar beet where it has the effect of deterring the growth of competing vegetation and poisoning predators [243]. The toxicity of azetidine-2-carboxylic acid has been reported for a variety of species, including poultry, rodents and humans [244, 245]. Accordingly, the 4-Maz could be the origin of the cytotoxic activity observed for the vioprolides. This could be a promising starting point for further SAR studies. Apart from the vioprolides there is only one group of bacterial metabolites containing an azetidine ring, namely the polyoxins produced by *Streptomyces cacaoi* [246]. Here, feeding studies [247, 248] as well as *in silico* genome analysis [249] revealed the formation of the polyoximic acid from isoleucin, which itself can be generated from methionine, threonine and glutamate. From these four amino acids, only L-threonine was fed to to the culture in this study, which did not result in elevated titers of either vioprolides A+B or C (the 4-Maz is incorporated in vioprolides A and C) (Fig. 3.13A+B). Additional experiments

feeding isoleucine, methionine and glutamate will have to be conducted to be able to investigate any effects on the product concentration.

The feeding of L-proline led to increased product concentrations of vioprolides C and D in both media (Fig. 4.1). Interestingly, the titer of vioprolide C could be increased 1.6-fold (in meat peptone medium) and 2.4-fold (in yeast extract medium), whereas the concentration of vioprolide D was only 25 % and 90 % higher, respectively. This is interesting because vioprolide D consists of two L-proline moieties, vioprolide C of only one (Fig. 1.7). Vioprolide B also contains one L-proline, but the concentration of vioprolides A+B increased only in meat peptone medium when L-proline was fed to the culture by 52 %. In yeast extract medium, the concentration of vioprolides A+B even decreased after addition of L-proline. Taking these facts into account, it seems as if the amino acid supplementation affects the building blocks at one position (L-pipecolic acid for vioprolides A+B, L-proline for vioprolides C+D) more than the ones incorporated at another position (4-Maz for vioprolides A+C, L-proline for vioprolides B+D). As 4-Maz has not been fed so far, this statement is purely hypothetical. To investigate the fate of the supplemented amino acids in general, it could be useful to add isotope-labeled amino acids [250]. This method helps to elucidate whether these amino acids are used for general cellular protein synthesis or for the biosynthesis of secondary metabolites. For the heterologous production of myxochromides in *Pseudomonas putida*::CMch37a it could be shown that the production was substantially enhanced through feeding of the precursor amino acids. Furthermore, the study revealed that the added amino acids are to a large extent directly incorporated in the product, due to an increased intracellular availability [251]. The observation that the distribution of derivatives generally differed between yeast extract and meat peptone medium could also be attributed to the varying supply of different amino acids in these two media components.

In this study, it could be shown that the derivative distribution of vioprolides heterologously produced by *M. xanthus* DK1622 can be controlled by

the supplementation of specific precursor amino acids. As some of the vioprolides are interesting candidates for drug-development, this method presents a simple way of producing the desired compounds in a multi-gram scale. But, as pointed out, more experiments are necessary to understand the nutritional behavior of *M. xanthus* DK1622 as well as the vioprolide biosynthesis in order to being able to optimize this process even further.

5 General Discussion and Outlook

In the last three decades, the number of new anti-infective drugs on the market has decreased drastically compared to the period of 1940-1980. One reason is the fact that pharmaceutical companies have resigned from natural product research because the number of new promising compounds was too low compared to the time and effort needed. With the beginning of the 1990s combinatorial chemistry was thought to fill the gap but effectively only two drugs discovered by these means have been introduced to the market. To overcome the current deadlock, so far neglected or underrepresented species have to be investigated more thoroughly for their potential as sources of novel anti-infective compounds. The order of Myxococcales consists of 7500 different strains that together produce as many as 100 compounds with different core structures and hundreds of derivatives. Most of these compounds have anti-bacterial, anti-fungal or cytotoxic activities, many of them having new modes-of-action. Besides the myxobacteria there are other producers of secondary metabolites living in the soil, like those of the genera *Chitinophaga* and *Flexibacter*. One of the main reasons why these bacteria have been neglected in the past are the low product titers that make production of pharmaceuticals economically non-viable. Additionally, the original producer strains have diverse nutritional requirements and the products are often unstable. These challenges need to be overcome for gliding bacteria to become producers of clinically relevant drugs. The three projects presented in this thesis deal with various aspects of development and optimization of fermentation processes to increase the accessibility to these valuable compounds.

The first project covered the production of the anti-MRSA compound family of elansolids by its native producer *Chitinophaga sancti* Fx7914. Bioreactor cultivations in complex media, as performed in the past, yielded only the non-reactive derivatives elansolid B1, B2, C1 and D2. But for medicinal chemistry studies investigating the SAR of the elansolids, a reactive derivative is needed that could be modified semi-synthetically. The use of a

chemically defined medium in combination with compound adsorption with the adsorber resin XAD16 enabled the production of elansolid A2 with titers of 55.3 mg L^{-1}. This fact shows that the media composition is a crucial aspect when optimizing biotechnological processes because it can affect not only the growth of the microbial strain and the overall yield but the derivative composition can be influenced as well. This holds also true for the second consideration of this project, namely the direct control of derivative composition by feeding of certain precursors that are incorporated into the compounds. This approach enabled the production of elansolid C1 through feeding of anthranilic acid with a concentration of 257.5 mg L^{-1} and a share of 85 % in the overall elansolid yield.

This shows the power of biotransformation and biosynthesis. Precursor-directed chemical synthesis of new elansolid derivatives was shown to be possible as well but with immensely lower yields. There are already medicinal chemistry studies underway that investigate the structure activity relationship of new elansolid derivatives with the aim of improvement of the physiochemical and pharmacokinetic properties of the molecules. If compounds with desirable properties are found, the protocol established in this study could be the basis for precursor-directed biosynthesis approaches that would allow the production of significant amounts of the new compounds necessary for further testing. Through the use of small-scale parallel cultivation systems the generation of different molecules would be possible as well. If simple precursor-directed biosynthesis did not yield the desired products, the next step in this direction would be a mutasynthesis approach where certain biosynthetic pathways were disrupted in the producer strain. Instead, different precursors could be fed that would be incorporated as building blocks for new elansolid derivatives by *C. sancti*. The main requirement for this method is the amenability of the strain for genetic manipulation which sometimes proofs difficult for gliding bacteria.

The other two projects dealt with the process optimization of the heterologous production of two different types of myxobacterial compound classes

with the model strain *Myxococcus xanthus* DK1622. The concentration of the α-pyrone antibiotic myxopyronin could be increased from 16.9 mg L^{-1} to 142.2 mg L^{-1} mainly due to adaptation of media composition and lowering of the cultivation temperature. The latter was identified to be a significant factor with the help of statistical experiment design (DoE). DoE presents a powerful tool to identify and optimize important factors that influence the output variable. In this case, only the first step of a DoE round, namely the identification of significant factors was carried out, as only the temperature was identified to be significant and could subsequently be investigated in a traditional experimental setup. For the DoE approach to uncover its full potential it is necessary to already have a basic understanding of the cultivation process to be able to identify errors and outliers. DoE is based on the mathematical calculation of a model that describes the influence of the input factors on the output factors. Since biological processes cannot always be described with simple correlations based on linear or quadratic equations, the expertise of the experimenter plays a significant role for the outcome using this statistical approach.
The fact that the product concentrations of vioprolides could be increased 10-fold through bioreactor cultivations compared to shake flask cultivations is striking. This illustrates the impact of up-scaling on the production process. The environmental conditions in shake flasks are often not ideal and cannot be controlled. Bioreactor cultivations on the other hand provide a controllable environment that can be adjusted to the distinct requirements and lead to reproducible results. Furthermore, the control of the derivative composition through feeding of precursors could be demonstrated. This fact is not be underestimated as most myxobacterial compounds are produced as a mixture of different derivatives of which in many cases only one is clinically relevant.

The aim of the heterologous expression of these two compound classes was the investigation of *M. xanthus* as an expression platform for natural products.

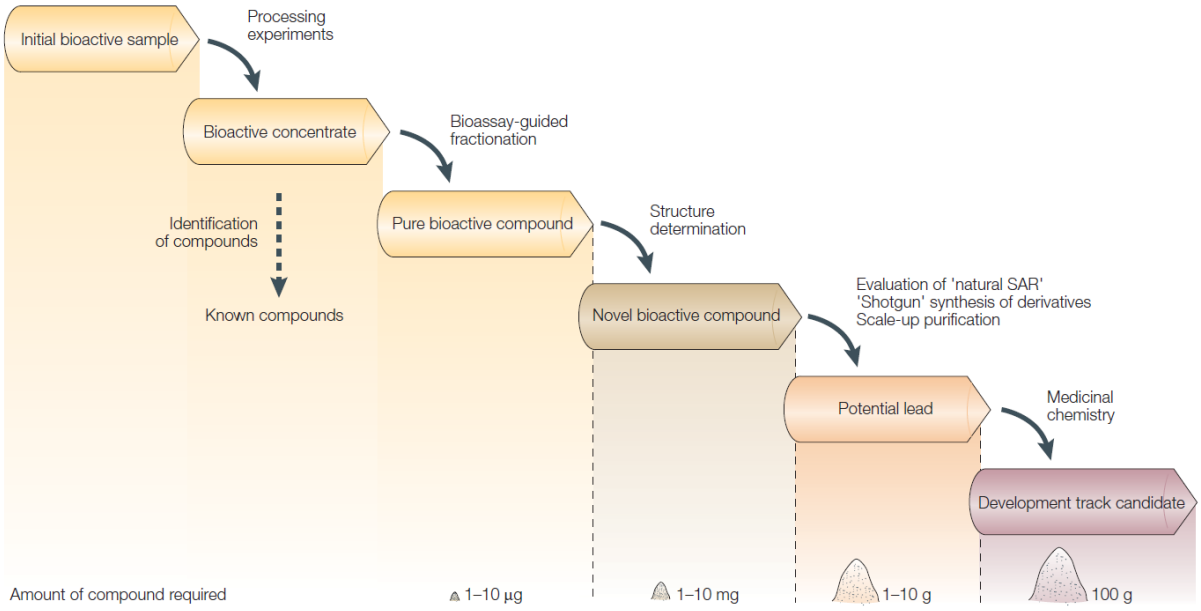

Figure 5.1: Amount of compound required for each stage of the natural product dicovery process [252].

As pointed out in chapter 1.5, the strain possesses the properties for expression and tailoring of PKS- and NRPS-derived compounds. The optimization of the production processes in the natural host for every product is tedious and in some cases not very successful. Having an expression platform for these compounds would facilitate the production and further development. The two biosynthetic gene clusters were cloned into the same place in the genome using the same technique to avoid any differences stemming from the integration. However, the production kinetics differed immensely between the two product classes. The α-pyrone myxopyronin A was produced during the exponential phase with the maximum concentration reached after 48 h at 30 °C cultivation temperature. The stationary phase was reached at the same time. The growth of *M. xanthus* DK1622 with the vioprolide biosynthetic gene cluster was generally slower, the stationary phase was reached after 96 h. Furthermore, the vioprolide production started in the late exponential phase with a maximum concentration reached after 240 h. Both gene clusters stood under the constitutive *tn5*-promoter, so the differences must have another origin that needs to be investigated in the future. Further-

more, the vioprolide concentration could not be increased by lowering the cultivation temperature, as for myxopyronin (data not shown). Therefore, biosynthetic gene clusters of other PKS- and NRPS-derived myxobacterial products should be cloned into *M. xanthus* to investigate if the differences originate from the differences in the biosynthetic machineries or if every product has individual production kinetics.

With the product titer improvement to the range of 100-500 mg L^{-1}, the amounts needed for further drug development stages can be easily reached (Figure 5.1). Commercial production of e.g. antibiotics however is estimated to reach 20-50 g L^{-1} [253]. These numbers make clear that the improvements made in this thesis can only be the first steps towards the introduction of myxobacterial compounds as marketable drugs. Besides the bioprocess engineering, especially mutagenesis and metabolic engineering approaches (as described in chapter 1.6.3) need to be employed to improve the yield further [254].

6 References

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